MANOMETRIC METHODS

LONDON

Cambridge University Press

FETTER LANE

NEW YORK · TORONTO

BOMBAY · CALCUTTA · MADRAS

Macmillan

TOKYO

Maruzen Company Ltd

MANOMETRIC METHODS

AS APPLIED TO THE MEASUREMENT OF CELL RESPIRATION AND OTHER PROCESSES

by

MALCOLM DIXON, M.A., PH

Rockefeller Lecturer in Biochemistı
in the University of Cambridge

With a Foreword by

SIR F. G. HOPKINS

President of the Royal Society

CAMBRIDGE

AT THE UNIVERSITY PRESS

934

CONTENTS

PREFACE

This book deals with a well-defined group of methods which, on account of their usefulness and adaptability, have come into extensive use in biological research. No general account of the theory and practice of these methods has previously appeared, although one or two articles dealing with certain special branches of the subject exist. It has consequently been necessary for anyone wishing to make use of the methods to collect the necessary information on points of technique from a number of scattered and frequently inadequate descriptions in the literature. Indeed, several important parts of the theory are nowhere dealt with in the existing literature; and many of the pitfalls which may entrap the inexperienced worker are nowhere mentioned, and must be discovered by experience. The need for a full account of the whole subject is therefore evident, and it is with the object of meeting this need that this book has been written.

It is intended essentially as a handbook for the laboratory, and my aim has been to supply in a convenient form just that information which is likely to be required by research workers using the methods. I have not attempted to give any account of the historical development of the apparatus and methods, or of those modifications which have proved unsatisfactory and are no longer in use. I have, however, endeavoured to give a full account of the apparatus and methods in use at the present time. The theory of the various manometers is discussed in detail, particularly in the case of the differential manometer, for which a full

treatment has not previously been given. The description of the experimental procedure has also been made as complete as possible, and attention has been directed to a number of practical points which have been found, during twelve years' personal experience of manometric work, to be important for the attainment of accuracy.

The symbols employed in the theoretical parts of this book are as far as possible those in most general use, and they are used consistently throughout. As however different authors often use different symbols, it is inevitable that in some cases formulae should appear here in slightly different dress from that which they wear in the original papers. Alterations have however been made as sparingly as possible, and it is hoped that no confusion will be caused thereby.

I should like to record my grateful appreciation of the kindness of Sir Frederick Hopkins in undertaking to write a foreword to this book.

M. D.

THE BIOCHEMICAL LABORATORY
CAMBRIDGE

August 1933

FOREWORD

That scientific progress depends largely on the development of new experimental methods is a commonplace of history. The methods described in this book are yielding in many fields of enquiry knowledge which in their absence would certainly have proved very difficult to obtain.

This is perhaps particularly true of knowledge concerning the respiratory activities of individual tissues. Physiology, from its earliest days as a progressive science, has sought for accuracy in measurements of that summation of tissue respiration which is observed in the gas exchanges of living animals and of man. A large body of accurate knowledge concerning this had been won by the end of the last century. Awakening interest, however, in the organisation of physiological functions and concern with the chemical details of intermediary metabolism stimulated endeavours to explore the respiratory activities of individual organs and tissues. Such efforts began, though they attained to no accuracy, during the use of the artificial perfusion technique as initiated in the laboratory of Ludwig. Much later more accurate estimations were made in the case of certain organs by the use of a method involving isolation of the circulation through the organ investigated, which was otherwise left intact and *in situ*. The oxygen and carbon dioxide were determined in samples of the inflowing and outflowing blood and since the velocity of the blood flow was known, information concerning the respiratory exchanges of the organ was obtained. The blood gases were estimated manometrically, the simple Barcroft-Haldane

and the Barcroft differential manometer being developed for the purpose. The convenience and potentialities of the manometric method, however, made such apparatus readily available for a direct study of isolated surviving tissues, provided that this technique could be so modified as to be suitable for measuring *rates* as distinct from *amounts* of gas exchange. The development of manometric methods on these lines has taken place chiefly at two centres: at Berlin, in the hands of O. Warburg and his school, using mainly the Barcroft-Haldane type of manometer, and at Cambridge, particularly in the Biochemical Laboratory, using mainly the differential type. Such methods so applied are now capable of a high degree of accuracy. The biological significance of the results depends of course upon the maintenance of physiological conditions for the tissues under study. Our knowledge of the factors which make for adequacy in media intended to support survival life has much advanced of late and leads to increased confidence in the significance of survival studies.

While the importance of manometry as applied to the respiration of isolated tissues and micro-organisms is rightly emphasised it should not be forgotten that the progress of individual chemical reactions can be accurately studied by its aid, in the case for instance of enzyme-catalysed oxidations, and also in many other cases. That it displays great accuracy as a micro-method is one of its advantages.

In the development of manometry in the Cambridge Biochemical Laboratory my colleague, the author of this book, has played the leading part. Dr Dixon was one of the first to use its methods with discrimination, and for some years he has given close and continuous attention to its growing technique. Apart from his own contributions to

this he has made himself very familiar with all the instruments and methods described in his book. He has used them all, and sedulously compared their relative merits. He writes, therefore, with authority.

Manometric methods have now been much employed in many laboratories, but it will be admitted, I think, by all who read the literature, that manometers have not always been used with due discrimination. It is a fact that the theoretical principles which underlie the behaviour of this or that form of manometer are less simple than those who give no thought to them are apt to realise, and the use of any form without due regard to these principles may involve errors of no negligible magnitude.

It is the great merit of this book that it provides an adequate and clear account of the theory involved in each of the methods described. To do this is part of its essential purpose and nowhere else has the task been attempted.

Part I of the book is devoted to a discussion of principles. Part II supplies guidance in detail for the use of the various methods which are now available. Much ingenuity has been displayed of late in the construction of new forms of manometric apparatus, each having merits of its own, and each perhaps possessing advantages over others for some particular purpose. As would be expected, however, those most recently described would seem to have advantages over earlier forms. Each method, however, calls for its own precautions and for attention to details which vary in importance from case to case. All such matters are fully dealt with in the pages which follow. I cannot indeed discover that any information necessary for the successful use of manometric method is here lacking.

I find much pleasure in writing this foreword to my

colleague's book because technical methods of great promise are here for the first time adequately appraised, and because of my faith that the book will be greatly welcomed by those who desire a guide to accuracy in their work.

F. G. HOPKINS

December 1933

INTRODUCTION

The most convenient and accurate methods available for following those reactions in which a gas is either absorbed or evolved are those usually called the manometric methods: i.e. methods in which the reaction is caused to take place in a closed vessel attached to some form of gauge-tube containing a liquid by means of which changes in the amount of gas in the vessel can be quantitatively measured. The measurement of the absorption of oxygen or the production of CO_2 by respiring cells, or by oxidation-reduction systems isolated from cells, is fundamental for the elucidation of the mechanism of cell respiration, and the manometric methods have been most extensively used for this purpose. Their usefulness does not however end here; for reactions involving the production or disappearance of acid or alkaline substances can also be followed manometrically by causing the reaction to take place in a bicarbonate buffer solution in equilibrium with a gas mixture containing CO_2. In this case the production of a given amount of acid will cause a corresponding amount of CO_2 to be given off, and this will be shown on the manometer. Many hydrolytic reactions for instance can be studied in this way.

Manometric methods can in fact be adapted to a great variety of purposes, and they are now widely and increasingly used in biological laboratories.

In Part I of this book an account is given of the construction and theory of the various types of manometers used, while Part II deals with the various methods by which they can be applied to the measurement of cell respiration and similar processes.

PART I

TYPES OF MANOMETER USED

The manometers used are of three main types. In the first type the gas in the vessel is kept at constant pressure by adjusting the liquid in a graduated tube connected with it, and the change in volume is read off from the tube. The principle of this type is that of the Haldane gas analysis apparatus: the Winterstein micro-respirometer is an example of this class. In the second type the vessel is attached to one end of a U-shaped manometer tube, the other end of which is open to the air. The liquid in the tube is adjusted to keep the gas at constant volume, and the change of pressure is read off, from which the amount of gas evolved or absorbed is easily calculated. This is commonly called the Warburg type of manometer on account of its extensive use by O. Warburg and his school, although due in the first place to Barcroft and Haldane. The third, or differential, type of manometer works neither at constant pressure nor at constant volume, both changing simultaneously. In this form the other end of the manometer tube, instead of being open to the air as in the Warburg type, is attached to a second vessel similar to the first, which has the effect of eliminating errors due to slight changes of temperature, etc. This is commonly known as the Barcroft type, as it has been developed mainly by him, although the principle was employed previously by E. Warburg for another purpose. These three types will now be considered in turn.

CHAPTER I

THE CONSTANT-PRESSURE TYPE OF RESPIROMETER

The principle employed will be clear from the diagram (Fig. 1). *A* is the reaction vessel, which contains the re-

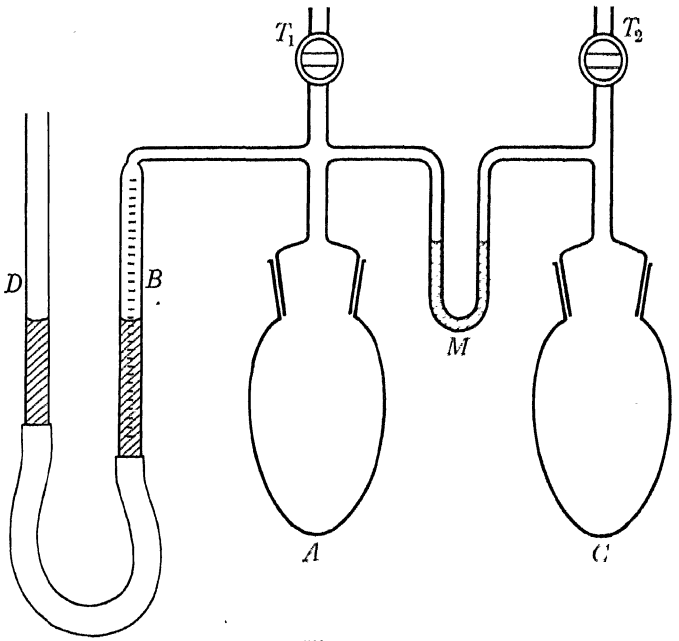


Fig. 1

spiring tissue or cell suspension. It is connected with a tube *B*, accurately graduated in hundredths of a c.c. *C* is a compensation vessel, similar to *A* and connected with it

through a manometer tube *M* which shows any difference of pressure between *A* and *C*. *A* and *C* are kept at the same temperature by immersion in a water-bath; the temperature of *B* should also be constant, though it need not be the same as that of *A* and *C* (e.g. *A* and *C* may be kept at 37° and *B* at room temperature). The method of taking the readings is as follows. The apparatus is placed in position in the water-bath with the taps T_1 and T_2 open, and the liquid in *B* is adjusted to some convenient point by raising or lowering the tube *D*. After sufficient time for the flasks *A* and *C* to attain the temperature of the water-bath, the taps are closed simultaneously, and the reading of *B* is accurately taken. After a given time *D* is again adjusted so as to bring the liquid in *M* to the same height on each side, and *B* is again read. The difference between the readings at the beginning and end of the period gives the volume of gas which has been absorbed in *A* during that period, measured at the temperature of *B* and the barometric pressure at the time of closing the taps. This must then be reduced to c.c. of dry gas at N.T.P. by multiplying by the appropriate factor, as given in Table I, p. 115.

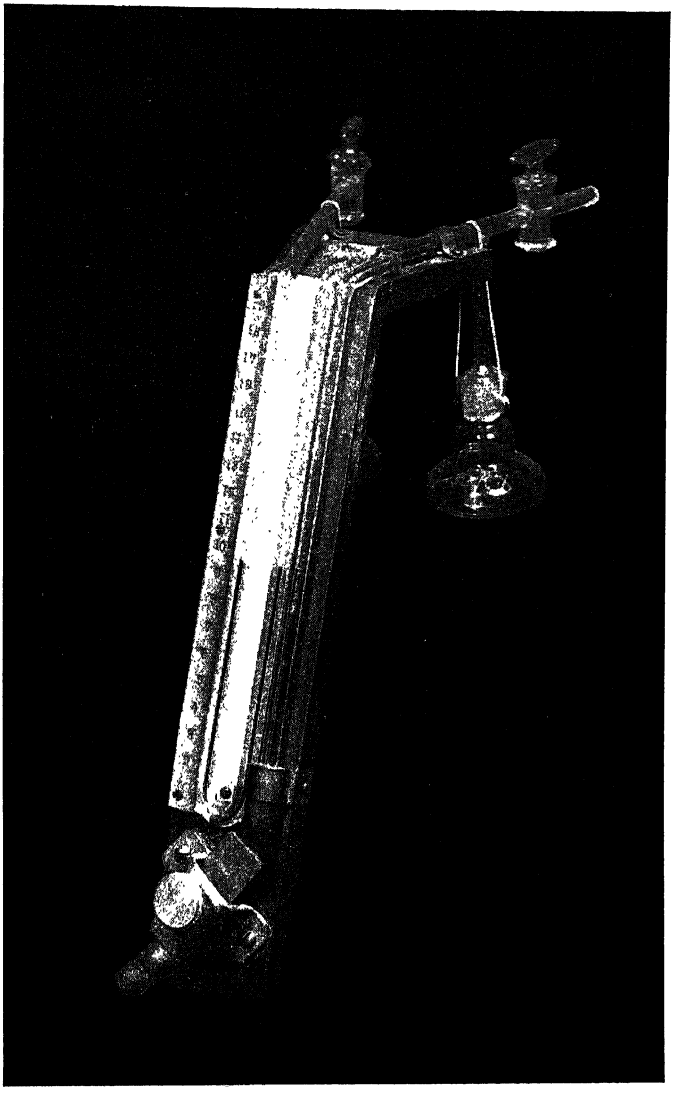
It will be obvious that if the vessel *C* and the manometer *M* were absent, and the readings were simply made by adjusting the liquid in tubes *B* and *D* to the same level, any slight changes in the temperature of the water-bath or the barometric pressure which might occur during the experiment would have a considerable effect on the readings. The function of the vessel *C* is to act as a control with which vessel *A* is compared, so that the apparatus is no longer affected by such variations of temperature or pressure.

The apparatus is perhaps best known in the form given

to it by Haldane (1921) for measurements on blood gases. In this form the tubes *B*, *D* and *M*, and the taps, are mounted on an upright board; the flasks *A* and *C* are immersed in a small water-bath, and they are connected with the rest of the apparatus by means of lengths of rubber tubing, so as to allow them to be shaken by hand when necessary. This instrument is, however, not very well adapted for measurements of cell respiration. It is excellent for measuring the total amount of gas absorbed or evolved by a reaction, but it is less suitable for measuring *rates* of reactions. Moreover, its form is inconvenient when several comparative measurements have to be made side-by-side. For these reasons it has been little used for work on respiration.

In the Winterstein (1912) micro-respirometer the U-tube *M* is replaced by a horizontal graduated capillary tube containing a single drop of liquid, which is brought to a given position in the tube before each reading is taken. The tube *D* is moreover replaced by a short length of rubber tube closed at the end and provided with a screw clamp by which it can be compressed. The liquid in tube *B* (which is also of a narrower bore) can thus be adjusted with greater accuracy.

In comparison with the other two types of respirometer, the constant-pressure type has not been very much used, owing to the fact that the available forms of the apparatus were not so convenient in practice as the Warburg and Barcroft manometers. It has, however, the advantage that the theory of the apparatus is very simple and that no "constant" needs to be determined. The author has very recently devised a form of constant-pressure manometer, here described for the first time, which he believes has



many of the advantages of all the forms.* This is illustrated in Fig. 2. It resembles in all respects an ordinary Barcroft respirometer as described later, except for the addition of the graduated 1 c.c. pipette shown on the right-hand side of the U-tube and a screw adjustment. The U-tube contains paraffin coloured with Sudan III, and the pipette contains mercury. Before each reading, the mercury is adjusted so that the liquid stands at the same height in each side of the U-tube. The volume changes read off from the pipette must of course be corrected to N.T.P. in the usual way. It is found that, provided direct handling of the pipette is avoided, its temperature remains sufficiently constant, even when the thermostat is at 37°. The method of use need not be further described, as it is similar in other respects to that of the Barcroft apparatus. It has the advantage over the latter that flasks of different volumes can be interchanged, the angle of tilt of the shaker (see below) can be altered, and the paraffin replaced by a fresh sample, without the necessity of recalibrating the apparatus: in fact, no calibration is necessary, except that the accuracy of the graduations on the pipette should be checked initially by weighing the mercury delivered in the usual way.

In order to test the apparatus, simultaneous measurements of oxygen uptake by a yeast suspension were made by means of the new apparatus and by Barcroft respirometers, and very close agreement was obtained.

This type of apparatus is less suitable for CO_2 measurements, as corrections for solubility in the liquid are then required.

* The respirometer of Adam (1920) works on the same principle, but its form is entirely different. It was devised for work on intact frogs' muscles, and it has not come into general use.

CHAPTER II

THE CONSTANT-VOLUME TYPE OF RESPIROMETER

The Warburg manometer (see Warburg, 1926) is illustrated in Fig. 3.* It consists of a U-tube of narrow bore (internal cross-section about 1 sq. mm.) the vertical sides of which are about 30 cm. long, and are accurately graduated in mm. This tube is provided with a rubber reservoir and screw-clamp arrangement by which the level of the liquid in the tube (Brodie solution† is generally used) can be adjusted. One end of the U-tube is open to the air, and the other end carries a tube as shown to which a suitable vessel can be attached by means of a ground joint. Through the tap *T* this can also be put into communication with the air. The lower end of the instrument is provided with a brass slide by which it can be attached to the shaking apparatus (see below), which is so arranged that when the manometer is in position the vessel is completely immersed in the water-bath.

The principle of the manometer is as follows. Before each reading the level of the liquid in that limb of the U-tube which is connected with the vessel is always brought by means of the screw adjustment to the same given point (say the half-way (150 mm.) mark). Only the level in the open limb is read, and the reading of the apparatus, which is the

* It is obtainable from Messrs C. Dixon and Co., 27, Devonshire Street, London, W.C. 1.

† The composition of Brodie solution is as follows: 500 c.c. water, 23 g. NaCl, 5 g. sodium tauroglycocholate, and a few drops of an alcoholic thymol solûtion. (10,000 mm. Brodie solution is approximately equivalent to 760 mm. Hg.)

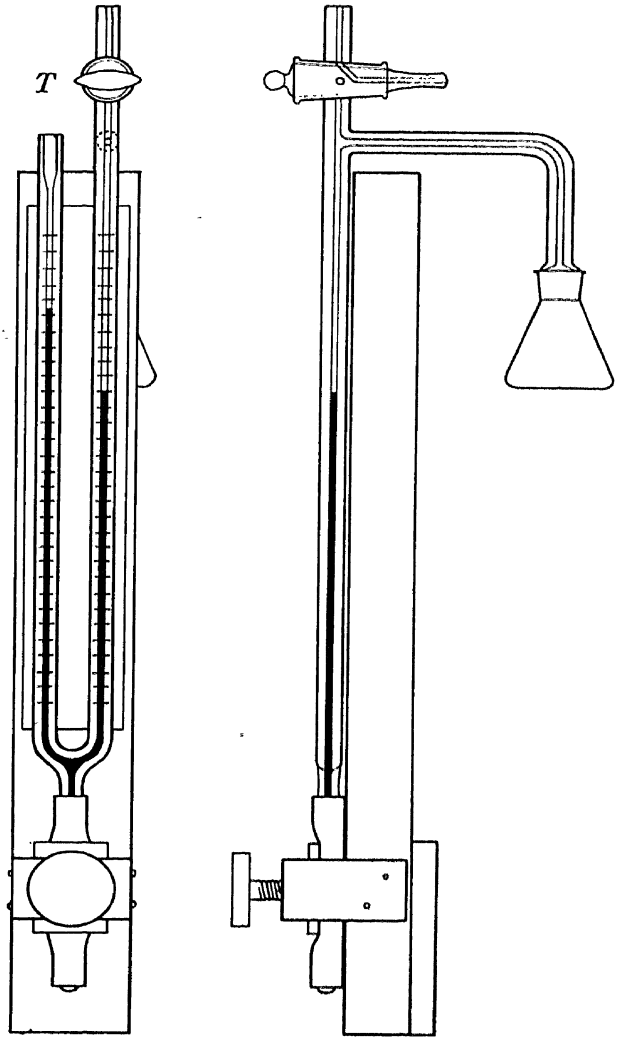


Fig. 3

difference of pressure between the interior of the vessel and the outer air, is obtained by subtracting 150 from the reading of this limb. From the reading of the manometer the amount of gas which has been evolved or absorbed in the vessel can be found in a manner which will be clear from the following account of the theory of the apparatus.

Theory of the Apparatus

The usual convention, to which we shall adhere throughout, is to consider all quantities of gas evolved by a reaction as positive and all quantities absorbed as negative. The readings are correspondingly considered as positive or negative according as they indicate an evolution or an absorption of gas. In this manometer therefore the reading is positive if the liquid rises in the open limb.

For clearness, we shall throughout express all volumes in c.mm., all readings in mm., all pressures in mm. of the liquid used in the manometric tube and all quantities of gas in c.mm. of dry gas at N.T.P.

Let us suppose that the vessel contains a certain amount of liquid, and that some reaction takes place in this resulting in the production of a definite amount of some gas; and that it is required to find this amount from the resulting reading of the manometer. The gas space in the vessel is assumed to be already filled with the gas in question at atmospheric pressure.

Let x = the amount of gas evolved in c.mm. at N.T.P. (If the gas is absorbed x will be negative.)

h = the corresponding reading of the manometer.

v_G = the volume of the gas space in the vessel (which must include the connecting and manometer tubes down to the 150 mark).

v_F = the volume of liquid (water, etc.) in the vessel.

T = the absolute temperature of the water-bath.

P = the initial pressure in the vessel (in general equal to the barometric pressure).

P_0 = the normal pressure (760 mm. Hg) (in mm. of manometric fluid). If D is the density of the fluid $P_0 = 760 \frac{13{\cdot}60}{D}$.

p = the vapour pressure of water at temperature T.

α = the solubility of the evolved gas in the liquid in the vessel (in c.mm. of gas (at N.T.P.) dissolved in 1 c.mm. of liquid when in equilibrium with a partial pressure of the gas equal to P_0).

Then initial amount of gas in gas space $= v_G \frac{273}{T} \frac{P-p}{P_0}$,

and initial amount of dissolved gas $= v_F \alpha \frac{P-p}{P_0}$.

Also final amount of gas in gas space $= v_G \frac{273}{T} \frac{P-p+h}{P_0}$,

and final amount of dissolved gas $= v_F \alpha \frac{P-p+h}{P_0}$

Now the total amount of gas finally present is obviously the amount initially present plus the amount x produced. Thus we have

$$\left(v_G \frac{273}{T} + v_F \alpha\right) \frac{P-p+h}{P_0} = \left(v_G \frac{273}{T} + v_F \alpha\right) \frac{P-p}{P_0} + x,$$

whence

$$x = h \frac{v_G \frac{273}{T} + v_F \alpha}{\qquad} \qquad \ldots\ldots(1).$$

Now it will be seen that the expression in square brackets remains constant for a given gas with any given instrument, provided the liquid volume and the temperature remain the same. This quantity is known as the constant of the apparatus (k). If we know its value we have only to multiply the reading by it, and we obtain immediately the amount of gas evolved in c.mm. of dry gas at N.T.P., which is what we require. Thus no further correction of any kind is needed:

$$x = hk.$$

The above equation (1) is equally valid if a second gas is present in the vessel in addition to the reacting gas: if, for instance, we have air in the flask instead of pure oxygen in measuring the oxygen uptake of a tissue. For the amount of nitrogen in the gas space remains constant throughout, and therefore cancels out from the equation; and since the gas volume is kept constant the partial pressure of nitrogen remains the same, so that the amount of dissolved nitrogen does not change. It is the partial pressure of the reacting gas (the oxygen) which changes, and it changes by an amount h, so that the amount of oxygen in solution changes as before by an amount $v_F \alpha \frac{h}{P_0}$. Thus the presence of the nitrogen does not affect the value of the constant. This explains why the solubility α in the equation is that of the reacting gas and not that of the gas mixture in the flask.

The value of the constant then depends on the nature of the gas absorbed or evolved; and since the two gases involved in respiration, O_2 and CO_2, have very different solubilities, the constant for oxygen may have an appreciably different value from that for CO_2 in the same apparatus.

Warburg has made ingenious use of this fact in his indirect method which is dealt with later.

Calibration of the Apparatus

The apparatus can be calibrated (i.e. the value of the constant can be determined) by three methods: (*a*) by calculation from the above formula (1), (*b*) by the Münzer and Neumann method, in which a measured amount of gas is added to or withdrawn from the flask by means of a graduated pipette, and the resulting reading of the manometer observed, and (*c*) by liberating or absorbing a known amount of gas in the vessel by means of a chemical reaction. The first method is the most accurate, and is the only one used to any great extent for Warburg manometers. The writer has compared the three methods and found very close agreement.

Of the quantities appearing in the expression for the constant given above, T and v_F are given by the conditions of the experiment, and α at temperature T is obtained from tables of physical constants (see p. 116). We must therefore determine v_G and P_0. The determination of P_0 involves merely a careful determination of the density D of the manometric liquid by the usual methods. v_G is obtained by subtracting v_F from the total volume of the flask. The most accurate way of determining the latter is as follows. A mark is made on the tube about an inch above the ground joint on to which the flask fits. The manometer is inverted (before introducing the Brodie solution), the tap closed and mercury poured into the neck to which the flask fits so as to fill the tubes from the 150 mark on the manometer to the mark made on the stem. The mercury is then run into a weighing vessel. The manometer is then restored to its normal posi-

tion, the flask filled with mercury and placed in position on the ground joint, the amount of mercury being adjusted so that it just rises to the mark on the stem when the flask is pushed home on its neck. This mercury is then added to the weighing vessel, its temperature immediately taken and the whole weighed. The weight of the mercury in mg., divided by the density of mercury at that temperature (see p. 115), gives the required volume in c.mm. The value of the constant can now be calculated with considerable accuracy.

The other two methods will be discussed in connection with the Barcroft apparatus.

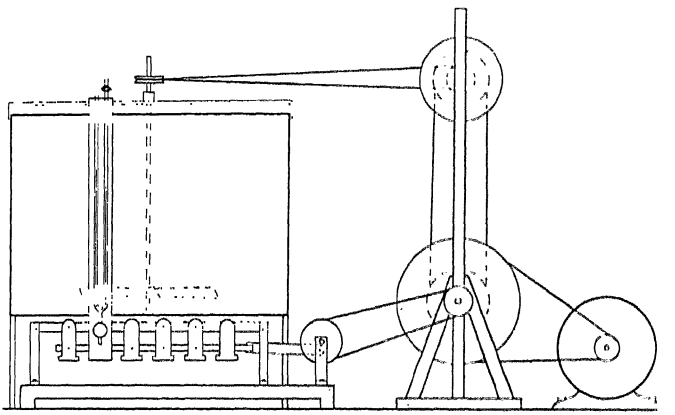
Thermobarometer

Warburg manometers are convenient for many purposes, but owing to the fact that one end of the manometer tube is open to the air they are very sensitive to slight changes in the barometric pressure or the temperature of the water-bath which may occur during an experiment. A change of a small fraction of a degree will alter the reading by several mm., and the barometer frequently changes sufficiently during an hour to alter the reading by 15–20 mm. or even more. It is therefore necessary to have not only a very efficient thermoregulator, but also an additional manometer to act as a thermobarometer. The latter is merely an extra Warburg manometer, the flask of which is left empty except for a little water, placed beside the experimental ones in the thermostat. Whenever a reading is taken this is also read, and its reading subtracted from those of the other manometers, in order to eliminate the errors due to changes in the external conditions. It is clearly not necessary to know the constants of this manometer, for, since the increase of

pressure produced by warming an enclosed volume of gas is the same whatever the volume of the containing vessel and since any change in the barometer acts equally on the open ends of all the manometers, any changes will affect the *readings* of all the instruments in the same way, even if their vessels are of different volumes. With this correction, the readings are usually significant to the nearest $\frac{1}{2}$ mm.

Thermostat and Shaking Apparatus

The water-bath and arrangement for shaking the manometers are as shown in Fig. 4. The same motor ($\frac{1}{8}$ H.P.) is



(*After* Warburg, 1926)

Fig. 4

used for stirring the water-bath and for shaking the manometers, but the shaker is provided with an idle pulley on to which the belt can be slipped, so that the shaking can be stopped when readings are to be taken without interrupting the stirring of the thermostat. Six manometers can be placed side-by-side in the shaker, and if a second shaker is

provided at the opposite side of the water-bath twelve instruments can be accommodated at one time. The shaker is adjusted so that the upper part of the manometers travels through about 5 cm., and the rate of shaking is usually 90–100 complete oscillations per minute. When readings are to be taken the shaker must be turned by hand until the manometers are exactly vertical, otherwise errors will be introduced. Also unless the line of sight is at right angles to the scale, parallax errors will occur. The instruments are provided with a strip of mirror behind the scales in order to prevent this.

Further details regarding the various types of flask and the use of the instrument as a respirometer are given in a later section.

Thermoregulator

For accurate manometric work an efficient thermoregulator is a necessity. The large toluene type usually supplied, though sensitive, reacts somewhat slowly owing to its large heat capacity. For work at 37°, better results are given by a small ether thermoregulator. As these do not seem to be generally known it may be useful to give details here. The construction is shown in Fig. 5. The tube contains mercury, and the small bulb contains a few drops of ether. At about 37·5° the ether vaporises and drives the mercury up to the jet, cutting off the gas supply. The instrument is extremely sensitive and acts very rapidly, and when once adjusted needs no attention. After two or three months' continuous use, however, the ether gradually tends to disappear: fresh ether is then readily introduced by pouring a small quantity into the wide tube, plugging the lower end of this by a glass rod with a piece of rubber tubing on its end, and inverting

the instrument. The ether then flows above the mercury into the bulb. The end of the jet should not be rounded in the flame, but should be ground; it should be placed cen-

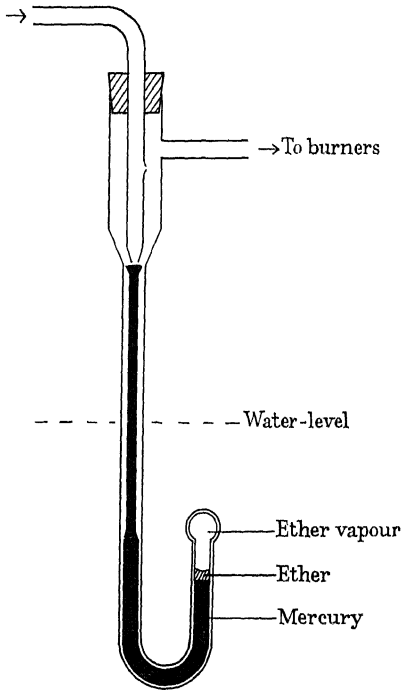


Fig. 5

trally slightly above the narrow part of the tube. A small hole is provided in the side of the inner tube as shown to act as a by-pass. The whole instrument should be quite rigidly mounted. It is possible to alter the temperature by one or

two degrees by adding or removing mercury. When first heating up, the ether may tend to superheat, but this is easily prevented by tapping gently when the correct temperature is reached.

The chief defect of this type of thermoregulator is that the temperature depends to a slight extent upon the barometric pressure. Under ordinary conditions, however, any changes of temperature due to this cause do not exceed a small fraction of a degree, and are quite negligible.

The writer has carried out a number of measurements with a sensitive Beckmann thermometer, and has found that with this type of thermoregulator the temperature remains constant within one hundredth of a degree for long periods, in both the Warburg and Barcroft type of thermostat. The temperature was moreover uniform throughout the water-baths within one fiftieth of a degree.

CHAPTER III

THE DIFFERENTIAL TYPE OF RESPIROMETER

The Barcroft apparatus (see Barcroft, 1908) differs essentially from the Warburg type in having one end of the manometer not open to the air, but connected to a second flask. This acts as a compensating vessel, so that the readings are no longer affected by changes of temperature or barometric pressure occurring during the experiment. For any changes of temperature will affect both flasks, and therefore both sides of the manometer, equally, and will therefore cancel out so far as the readings are concerned; and, since the apparatus is now a closed system, changes of external pressure can no longer affect it. A second point of difference is that the manometer is a simple U-tube, no screw adjustment for the liquid is provided, and hence the liquid moves in both sides of the tube, the reading of the apparatus being as before the difference between the readings of the two sides. These differences make the theory of the Barcroft apparatus somewhat more complicated than that of the Warburg apparatus, for both the pressures and the volumes of the gas in both vessels change.

Before working out the theory of the apparatus it is necessary to define quite clearly what is meant by the apparatus constant, because much confusion is caused by the fact that it has not hitherto been clearly recognised that two distinct kinds of constant are in common use at the present time, some workers using one and some the other. The first constant, which may be called k, is completely analogous to

the Warburg constant already mentioned; i.e. on multiplying the reading of the apparatus by it one obtains the corresponding amount of gas expressed in c.mm. of gas at N.T.P. $k=\frac{x}{h}$. This is the constant which is used by most workers on respiration, etc. But there is a second constant also in use, which may be called k'. On multiplying the reading by this the result is obtained in c.mm. of moist gas at the temperature and pressure of the experiment, and requires subsequent correction to N.T.P. $k'=\frac{x'}{h}$, where x' represents the volume of gas under the conditions of the experiment. This constant is used by many of those who work on blood gases, and by a few of those who work on respiration. The existence of these two constants in common use, without clear recognition of the fact, has led to considerable confusion; and there are cases in which k' has been used and no correction to N.T.P. made, and also cases in which k has been used and the results subsequently corrected, giving erroneous values. It would be desirable to suppress the use of one of these constants altogether, and for the following reasons it is here suggested that k alone should be used. (*a*) The quantity which we wish to measure is the amount of gas reacting, expressed in c.mm. at N.T.P. The volume which this amount of gas would occupy at the temperature of the water-bath and at the atmospheric pressure at the time of the experiment is of little interest. It is therefore logical to use that constant which gives the required result directly and without subsequent correction. (*b*) The theory of the apparatus shows that k varies approximately inversely as the temperature but is independent of the initial (barometric) pressure, while k' is independent of

the temperature and varies approximately inversely as the initial pressure. Now we work usually at constant temperature but not at constant barometric pressure, and it is therefore much more convenient to use that constant which does not depend on the pressure. In what follows k' will not be used.

Theory of the Apparatus

The complete theory of the Barcroft apparatus has not previously been published, although a number of partial treatments have been given from time to time. The most complete treatment is that given by Warburg (1926), and the following account is based on this, though with several modifications and extensions. We may first work out the complete expression, and then see how far this can be simplified by the omission of terms without loss of accuracy.

Let us suppose that the apparatus (represented diagrammatically in Fig. 6) is filled with a given gas and that a certain volume of liquid (water) is present in both flasks. Let an amount x of the gas be produced by some reaction in the right-hand flask. (It is usual to use the right-hand flask as the reaction vessel and the left-hand flask as the compensation vessel.) If the resulting manometric reading is h, the volume of the gas space in the right-hand side of the apparatus will increase by an amount $A\frac{h}{2}$, and that in the left-hand side will similarly decrease by an amount $A\frac{h}{2}$, where A is the area of cross-section of the manometer tube in sq. mm.

In what follows the same symbols as before will be used, those with dashes referring to the compensation vessel and those without to the reaction vessel: e.g. v_G' means the

initial volume of the gas space in the left-hand flask, including of course the tubes to which it is attached as far as the centre (100 mm.) mark on the scale, at which the mano-

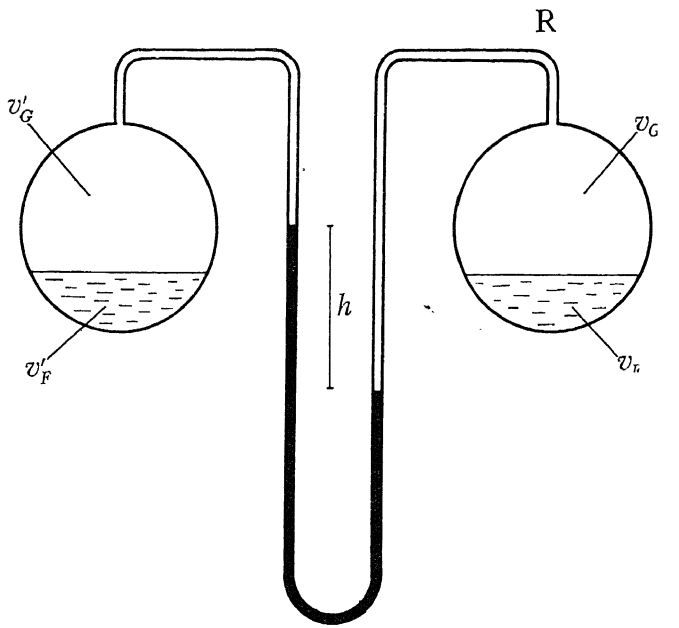


Fig. 6

metric liquid normally stands when the reading is zero. The treatment falls into three parts, concerned with the right-hand flask, the left-hand flask and the U-tube respectively.

(*a*) *The right-hand flask.* The amount of gas in the gas space initially is as before $v_G \frac{273}{T} \frac{P-p}{P_0}$, and it is easily seen

that the final amount of gas in the gas space will be $\left(v_G+\frac{Ah}{2}\right)\frac{273}{T}\frac{P-p+\Delta P}{P_0}$, where ΔP is the increase of pressure in the vessel. (ΔP is not equal to h, owing to the fact that the pressure rises in the compensation vessel also.) Since the pressure of the gas increases by ΔP, the amount dissolved will increase by an amount $v_F\alpha\frac{\Delta P}{P_0}$. Now of the amount x of gas produced, the amount just mentioned will go into solution, and the remainder will be given by the difference between the initial and final amounts in the gas space; x will therefore be the sum of these two quantities, and we have

$$x=\left(v_G+\frac{Ah}{2}\right)\frac{273}{T}\frac{P-p+\Delta P}{P_0}-v_G\frac{273}{T}\frac{P-p}{P_0}+v_F\alpha\frac{\Delta P}{P_0},$$

whence $$x=v_G\frac{273}{T}\frac{\Delta P}{P_0}+\frac{Ah}{2}\frac{273}{T}\frac{P-p+\Delta P}{P_0}+v_F\alpha\frac{\Delta P}{P_0}$$

and $$x=\Delta P\left(\frac{v_G\frac{273}{T}+v_F\alpha}{P_0}+\frac{A}{2}\frac{273}{T}\frac{(P-p)\frac{h}{\Delta P}+h}{P_0}\right) \quad \ldots\ldots(2).$$

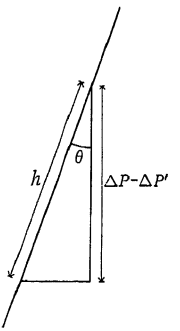
(*b*) *The left-hand flask.* Since the amount of gas produced in the left-hand flask is zero, we have by similar reasoning, writing $\Delta P'$ for the increase of pressure in this vessel and remembering that here the volume diminishes instead of increasing,

$$0=\Delta P'\left(\frac{v_G'\frac{273}{T}+v_F'\alpha}{P_0}-\frac{A}{2}\frac{273}{T}\frac{(P-p)\frac{h}{\Delta P'}}{P_0}\right)$$

whence $$v_G'\frac{273}{T}+v_F'\alpha=\frac{A}{2}\frac{273}{T}\frac{(P-p+\Delta P')h}{\Delta P'}$$

and
$$\Delta P' = \frac{\frac{A}{2}\frac{273}{T}(P - p + \Delta P')}{v_G'\frac{273}{T} + v_F'\alpha} \cdot h \qquad \ldots\ldots(3).$$

(*c*) *The manometer tube.* The Barcroft apparatus is usually made with its manometer tube not vertical but tilted backwards at an angle of about 20°, in order to facilitate reading, and the shaking apparatus is designed to hold them at this angle.* Since it is the vertical difference in level between the menisci in the two sides of the tube which measures the difference of pressure, and since h is measured along the sloping scale, it is necessary to multiply h by $\cos\theta$ to obtain the difference of pressure, where θ is the angle of the manometer with the vertical. (See the diagram, which represents a side view of the manometer tube.) As the pressure in both flasks was the same initially, and as the pressure has increased by ΔP and $\Delta P'$ in the right and left sides respectively, the difference of pressure is clearly $\Delta P - \Delta P'$, so that we have for the manometer



$$h\cos\theta = \Delta P - \Delta P' \qquad \ldots\ldots(4).$$

We may now bring these three equations together as follows. From (4),

$$\Delta P = h\cos\theta + \Delta P',$$

* The writer does not consider this desirable, as a slight change, accidental or otherwise, in the angle of the shaker will produce an alteration in the value of the constant. But in laboratories equipped with manometers and shakers of the tilted type it will probably not be considered worth while to remodel the whole equipment so as to make the manometers vertical.

and substituting from (3) we have

$$\Delta P = h\left(\cos\theta + \frac{\frac{A}{2}\frac{273}{T}(P-p+\Delta P')}{v_G'\frac{273}{T}+v_F'\alpha}\right)$$

Substituting this value in (2) we finally obtain

$$x = h\left(\cos\theta + \frac{\frac{A}{2}\frac{273}{T}(P-p+\Delta P')}{v_G'\frac{273}{T}+v_F'\alpha}\right)\frac{v_G\frac{273}{T}+v_F\alpha}{} + \frac{A}{2}\frac{273}{T}\frac{(P-p)\frac{h}{\Delta P}+h}{P_0} \quad ..(5).$$

It will be seen that this complete expression for the "constant" contains four terms: the first is due to the tilt of the manometer, the second expresses the effect of the compensation vessel, the third is the main term which appears also in the constant of the Warburg manometer, and the fourth represents the effect of the volume change due to the movement of the manometric liquid. It will be seen also that owing to the appearance of several minor terms involving h and $\Delta P'$ the value of the "constant" changes slightly with h: i.e. the reading is not exactly proportional to the amount of gas evolved over the whole range of the scale. It will be shown, however, that these terms are negligible under the usual conditions.

Simplification of the Formula

This expression can be considerably simplified without loss of accuracy by the omission of negligible terms, as we shall now show by applying it to the ordinary type of Barcroft

apparatus, in which v_G is about 40,000 c.mm., v_F usually 3000 c.mm., A somewhat less than $\frac{1}{2}$ sq. mm. (it should not greatly exceed this value except in special cases), and the length of the scale 200 mm. Working out approximately the values of the four main terms, we find that the value of the second is about 1/15th of that of the first, and the fourth also about 1/15th of the third. Thus an error of as much as 15 per cent. in the second or fourth terms will only produce a 1 per cent. error in the constant. From equation (3) we find that $\Delta P'$ is somewhat less than 1/10th of h, and inserting this value in equation (4) we see that ΔP only differs from h by 2 or 3 per cent., so that we can put $\frac{h}{\Delta P}$ equal to 1. Also h cannot exceed 200 mm., and therefore both h and $\Delta P'$ are negligible in comparison with P in the correction terms, for P is of the order of 10,000 mm. Further, except at high altitudes, P will be equal to P_0 within less than 5 per cent.; and if we avoid liquids more volatile than water and work at temperatures below 50°, p will be less than 10 per cent. of P (it will be shown below that effectively p is a good deal smaller than this), and therefore if we write P_0 instead of $P-p$ the error introduced will not exceed 1 per cent. in the whole constant, even in the most unfavourable case. Finally $v_F'\alpha$ in the second term does not exceed 5 per cent. of v_G', even with a very soluble gas such as CO_2, and it may therefore be omitted. We thus obtain the following simplified expression:

$$x=h\left(\cos\theta+\frac{AP_0}{2v_G'}\right)\left(\frac{v_G\frac{273}{T}+v_F\alpha}{P_0}+\frac{A}{2}\frac{273}{T}\right) \quad \ldots(6).$$

It should be clearly recognised that in order to make these simplifications A must be small (less than $\frac{1}{2}$ sq. mm.). If

A much exceeds 1 sq. mm. errors of several per cent. may be introduced by using the simplified formula under certain conditions.

Two assumptions have been made in the above treatment. The first of these is that the whole of the apparatus is at the temperature T. Actually only the flasks themselves are at this temperature; the manometer tube is at room temperature and the top tubes of the apparatus are at some intermediate temperature. The effect of this is greatly to reduce the errors introduced by the use of formula (6). It is unnecessary to give the full equations here: it will be sufficient to point out that as the manometer is at room temperature (say T_3) the space $A\frac{h}{2}$ which is added to or subtracted from the gas volumes by the movement of the liquid is at room temperature, so that in the second and fourth terms of equation (5) we should really write $\frac{A}{2}\frac{273}{T_3}$ instead of $\frac{A}{2}\frac{273}{T}$. Also for the same reason p should be the vapour pressure at room temperature instead of that at T, so that it becomes much smaller and its omission from the formula is still more justifiable. This is particularly the case, as it happens that even the small error due to this omission is practically neutralised by writing T for T_3, as is done in formula (6). Actual calculation shows that for values of T between 15° and 45° the error in the values of the second and fourth terms due to neglecting p and writing T for T_3 is less than 5 per cent., corresponding to an error of well below $\frac{1}{2}$ per cent. in the constant.

The fact that the top tubes (whose volume may be called v_2) are at an intermediate temperature (T_2) is expressed in the equation by substituting $v_1\frac{273}{T}+v_2\frac{273}{T_2}$ for $v_G\frac{273}{T}$,

where v_1 is the volume of the gas space in the flask excluding the top tube. As however the volume of the tube does not exceed 1/50th of that of the flask attached to it, the error introduced will be less than 0·1 per cent., and may safely be neglected.

The second assumption which has been made is that the flasks were initially filled with the reacting gas itself. The admixture of a second gas (e.g. nitrogen) will leave the value of the constant quite unaffected, unless this second gas is very soluble. The presence of any considerable proportion of a soluble gas such as CO_2 will have a very slight effect on the oxygen constant for the following reason. When oxygen is evolved in the reaction vessel the manometric liquid moves downwards on this side, increasing the volume. The partial pressure of the CO_2 in the vessel is thereby slightly diminished, and a small amount of dissolved CO_2 will pass into the gas space and so affect the manometer. Calculation shows, however, that even with a gas mixture containing 10 per cent. of CO_2 the error due to this cause is less than 0·1 per cent., and therefore quite negligible.

The conclusion to which we are led by this discussion is, then, that with the usual form of Barcroft apparatus the constants are given by the simplified equation (6) with an accuracy of at least 0·5 per cent. As the other errors inherent in manometric work usually amount to about 2 per cent., the determination of the constant is probably the most accurate part of the technique. Considerable departures should not be made from the usual dimensions of the apparatus without determining their effect on the validity of the formula.

Calibration of the Apparatus

The same three methods which were mentioned in connection with the Warburg manometer are available for determining the value of the constant of the Barcroft apparatus: namely, calculation from the formula, the Münzer and Neumann method, and the chemical method. The first method is the best in the writer's opinion; but owing to the absence of a satisfactory account of the theory of the apparatus some doubt has hitherto been felt as to the validity of the various formulae which have been proposed, and most workers have preferred to use the Münzer and Neumann method. The writer has compared these two methods carefully and found very close agreement (to about $\frac{1}{2}$ per cent.). Protocols are given in Appendix I.

In order to calculate the constant from equation (6) we must measure θ, A, the volumes of the left- and right-hand flasks plus their respective connecting tubes as far as the 100 mm. marks on the scales, and the density of the manometric liquid. The other quantities are known. θ must be carefully measured, and it is advisable to do this for each position in the shaker to ensure that there is no variation. The measurement is best made by placing a manometer in position, placing the straight edge of a piece of card against the scale, and ruling a vertical line on the card by means of a T-square or other device. A is measured by introducing into the U-tube a drop of mercury of such a size that it fills about 100 mm. of the tube. The length of the mercury thread is measured in four positions in the tube in order to test the uniformity of the bore, and the volume of the mercury then obtained by weighing. A is then given by dividing the volume by the average length. The bore should be

reasonably uniform: if large variations occur, surface tension effects may produce errors of 1 or 2 mm. in the readings. The volumes of the flasks and top tubes are measured with mercury in exactly the same way as already described for the Warburg apparatus, and v_G and v_G' obtained as before by subtracting v_F from these volumes. P_0 is obtained from the density of the liquid as before. k can now be calculated.

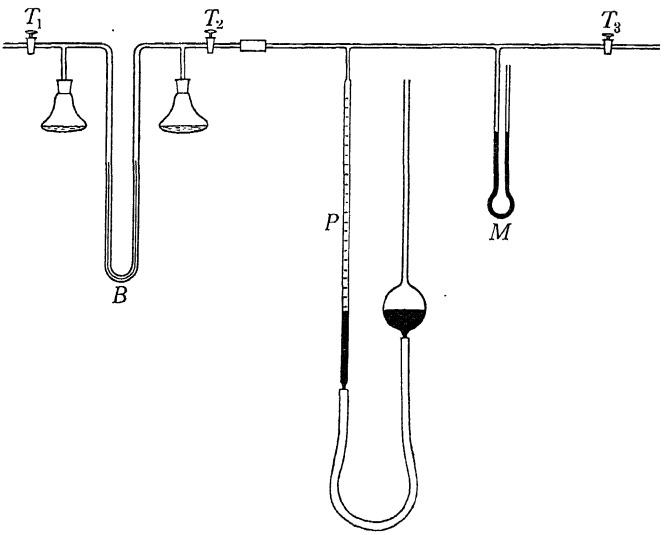


Fig. 7

As the Münzer and Neumann (1917) method is very widely used it will be described in some detail. An improved form of the arrangement used is represented diagrammatically in Fig. 7. The Barcroft apparatus is represented at *B*, *P* is a graduated 1 c.c. pipette and *M* is a separate manometer tube containing paraffin and open to the air at

one end. The flasks of the Barcroft apparatus and the pipette *P* are completely immersed in a glass-fronted water-bath. The right-hand side of the Barcroft apparatus is connected with *P* and *M* by means of narrow-bore glass tubing, short lengths of thick-walled rubber tubing being used to connect up where necessary. The graduations of *P* must be checked previously by calibrating it with mercury in the usual way. It contains dry mercury, the level of which can be accurately adjusted by raising or lowering the reservoir shown, which is provided with a fine adjustment. It has been found preferable to use mercury rather than water, as the accuracy of the results may be slightly affected by the film of water which remains on the sides of the pipette when the level is lowered.

The procedure is as follows. Place in the flasks of the Barcroft apparatus an amount of water equal to that which will be used in the experiments, place the apparatus in position in the water-bath and shake for a short time in order that the gas and liquid phases may come into equilibrium. Then connect up to the calibrating apparatus, and allow to stand for about 15 min. with all three taps open so that the temperature may become quite steady. Care should be taken that the angle of the manometer of the Barcroft apparatus with the vertical is exactly the same as that which it has when it is in position in the shaking apparatus. During this period the air in the pipette should be mixed with that in the right-hand flask once or twice by closing tap T_3 and raising and lowering the mercury several times. At the end of the period close taps T_1 and T_3, and take the readings of the Barcroft manometer and of *P* (the latter in c.mm.). Now lower the mercury in *P* so as to withdraw some gas from the right-hand flask, close tap T_2, and adjust the

mercury so as to bring the manometer M exactly level once more. Read the Barcroft manometer and the pipette again. Then the difference between the two readings of P gives the amount of gas which has been withdrawn from the Barcroft flask, measured at the temperature of the water-bath and at atmospheric pressure (since the manometer M is always levelled before reading P). We may call this volume x', as on p. 21, and the resulting reading of the Barcroft manometer (corrected for any slight initial difference in level) may be called h'. Then if we divide x' by h' we obtain k' as previously defined. To obtain k, x' must be corrected so as to represent c.mm. of dry gas at N.T.P. (i.e. x) and this divided by h'. After the second reading tap T_2 should be opened, the mercury adjusted so as to bring the Barcroft manometer to its initial reading, and P again read. This reading should be the same as the initial reading: any appreciable change indicates either a leak of gas or a change of temperature during the procedure. The whole process should be repeated five or six times, and the mean value taken. The individual values of k (or k') do not usually differ from one another by more than 1 per cent.

Thus if the change in the pipette reading is x', the resulting Barcroft reading is h', and the suffix c refers to the conditions of calibration:

$$k_c = \frac{x'}{h'} \frac{273}{T_c} \frac{P_c - p_c}{P_0}.$$

Both k and k' apply only when the Barcroft apparatus is used at the temperature and pressure at which it was calibrated. For instance, k expresses the relation between the amount of gas reacting (in c.mm. at N.T.P.) and the resulting reading of the manometer *when the apparatus*

is used under the conditions of calibration. This has been indicated by writing k_c to represent k for use at the conditions of calibration. Now we have seen that k varies with the temperature at which the apparatus is used, so that if the temperature T at which the experiments are to be carried out differs from T_c it is necessary to correct k_c for use at this temperature. (What applies to k with respect to temperature applies equally to k' with respect to barometric pressure.) In the case of the constant for oxygen under normal conditions this is readily done, as will now be shown, but in the case of the CO_2 constant it involves so much trouble that it is better to calculate all CO_2 constants directly from the formula. In any case, this method is not well adapted for the accurate determination of CO_2 constants, owing to the rapid diffusion of CO_2 through the rubber connections.

Consider equation (6). In the third term $v_F\alpha$ in the case of oxygen and with the usual 3000 c.mm. of liquid in the flask will not exceed 100, which is just negligible in comparison with v_G (which is about 35,000). (This means incidentally that the constant for oxygen is identical with the constant for air, which is actually what has been determined by the above procedure.) Now if we neglect $v_F\alpha$ it is clear from the formula that the whole constant becomes exactly proportional to $\frac{1}{T}$. It follows that k (the constant for use at T) is given by

$$k = k_c \frac{T_c}{T},$$

or

$$k = \frac{x'}{h'} \frac{273}{T} \frac{P_c - p_c}{P_0}.$$

The results obtained by the use of this constant do not require any further correction. The complete equation is in fact

$$x = h.\frac{x'}{h'}\frac{273}{T}\frac{P_c - p_c}{P_0}$$

It was mentioned above that some workers prefer to use k' and to correct the results to N.T.P. subsequently. This leads to the same final result, as may easily be shown. k' does not vary with T, but is inversely proportional to the gas pressure. Thus having obtained k_c' $\left(=\frac{x'}{h'}\right)$ as above described, it is unnecessary to apply any correction for change of temperature in order to obtain k' for the experimental conditions; but it is necessary to correct for any difference in barometric pressure or vapour pressure between the calibration and experimental conditions. k' is therefore given by

$$k' = \frac{x'}{h'}\frac{P_c - p_c}{P - p}.$$

The experimental reading h is now multiplied by this, and the resulting volume then corrected to N.T.P. to give x:

$$x = h.\frac{x'}{h'}\frac{P_c - p_c}{P - p}\frac{273}{T}\frac{P - p}{P_0},$$

or

$$x = h.\frac{x'}{h'}\frac{273}{T}\frac{P_c - p_c}{P_0}.$$

This is identical with the result obtained by the use of k.

It should be clearly understood that this part of the treatment assumes that the term $v_F\alpha$ can be neglected, which is not the case for soluble gases such as CO_2. In these cases the first method of calibration must therefore be employed.

The direct chemical method of calibration has not been very widely used as it is not as accurate as the other methods. (The maximum degree of accuracy attainable is in practice about ± 2 per cent.) The CO_2 constant can be obtained by liberating CO_2 in the flask by mixing known amounts of acid and bicarbonate. For the oxygen constant, the evolution of oxygen on mixing H_2O_2 with $KMnO_4$, or the absorption of oxygen by pure cysteine plus a trace of ferrous sulphate, may be used. The only advantage of this method is that the constants are obtained directly under the actual conditions of the experiments with only the simplest calculation (see Barcroft, 1914).

Details of the Apparatus

The Barcroft respirometer itself is illustrated in Fig. 8. The form shown is that of Dixon and Elliott (1930), which is that now generally employed for measurements of respiration. The earlier type had T-taps at the junction of the vertical and horizontal tubes instead of the taps shown. These were liable to leak or to become blocked, and the substitution of the taps shown effected a great improvement in the reliability of the apparatus. The U-tube is mounted between two scales graduated in mm., and a strip of squared paper is mounted behind it with the rulings in line with the graduations of the scales. The U-tube itself is not graduated. With this arrangement no difficulty is usually experienced in making readings accurate to 0·1 mm., and as the apparatus is unaffected by any slight changes which may occur in the environment the readings are actually significant to about this extent. The accuracy of the rulings of the squared paper must of course be tested, as specimens are occasionally met with which have an error of several per cent. The mano-

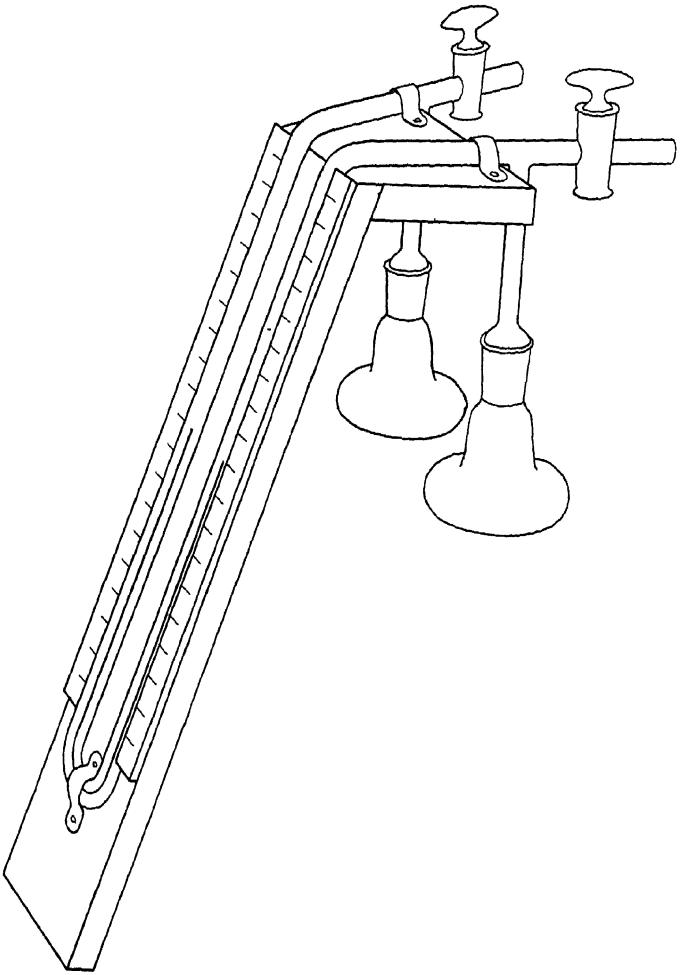


Fig. 8

meter contains ordinary paraffin fairly deeply coloured with Sudan III: clove oil, which was formerly used, is far too viscous. The best way of filling the manometer is to invert the apparatus and to run the paraffin into one of the angles at the top of the U-tube by means of a capillary pipette. In this position the amount of paraffin is easily adjusted so that it will fill the tube up to the 100 mm. mark approximately. After allowing it to run down into the U-tube, the top tube is dried out by means of a pipe-cleaner to prevent the subsequent formation of drops in the upper part of the tube. The flasks, which are of course numbered, are now made with standard ground joints, and it is convenient to have some spare flasks in case of breakage.*

Thermostat and Shaking Apparatus

The water-bath and shaking apparatus used with Barcroft manometers are shown in Fig. 9. It consists of an elongated water-bath provided with a thermoregulator and stirrer. The latter is either a bent glass tube rapidly rotated or a small four-bladed propeller. The front of the thermostat carries a brass frame provided with four or six screw clamps for holding the manometers and supported on two wheels so that it can be shaken longitudinally without much friction. An adjustment is provided for varying the angle of tilt of the manometers. The frame is shaken at a rate of about 120 complete oscillations per minute, the distance of travel being about 2·5 cm. A 1/30 H.P. motor is used to drive both the stirrer and the shaker, and a friction clutch, idle pulley or some similar device should be provided whereby the shaking can be interrupted for taking readings without

* The apparatus is obtainable from Messrs W. G. Flaig and Sons, 57, Hatton Garden, London, E.C. 1.

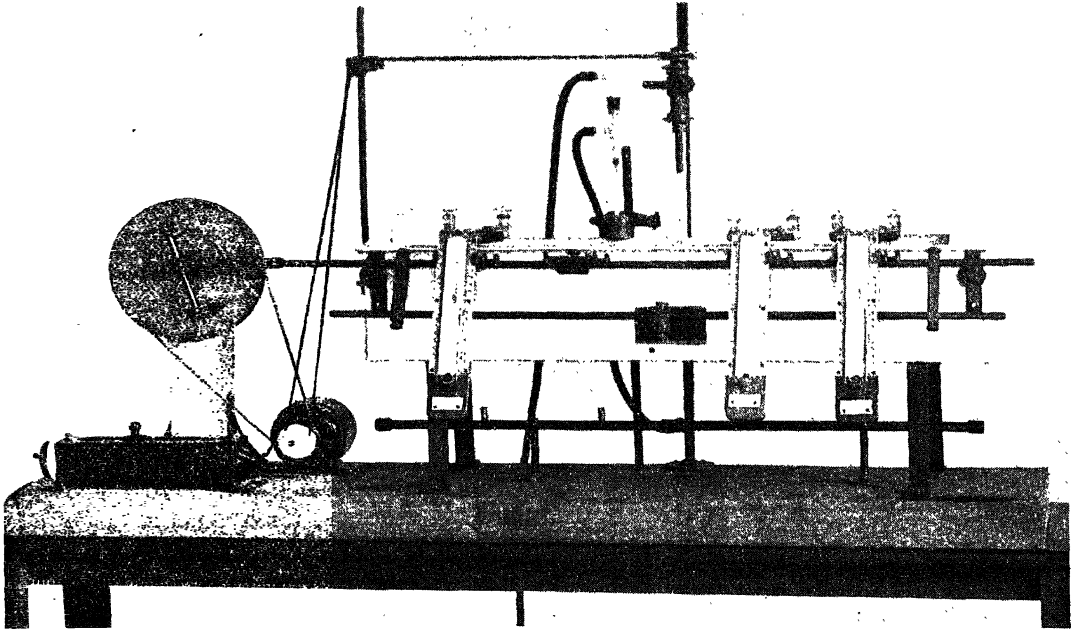


Fig. 9

stopping the stirrer. It is convenient to mount the whole outfit on a thick board, so that it can be moved from place to place.

The bath is heated by three or four burners spaced along its length as shown.

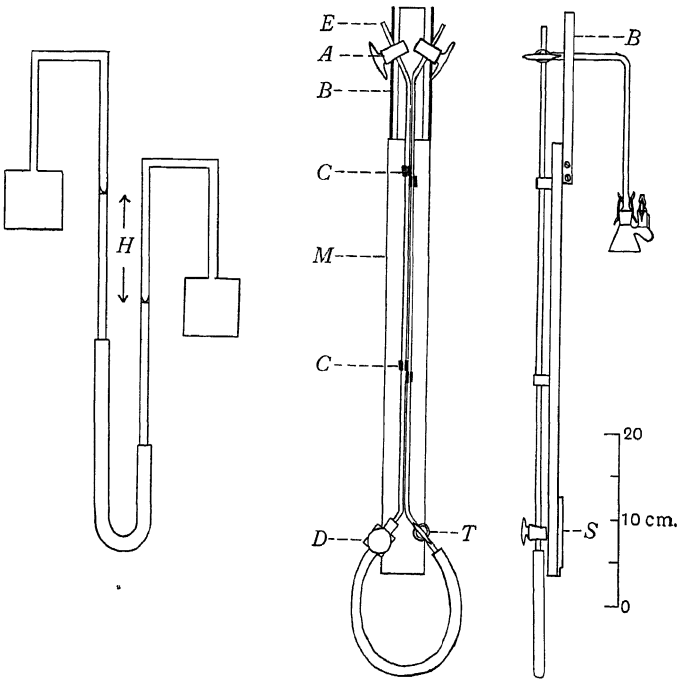
Further practical details relating to the use of the Barcroft apparatus for the measurement of respiration will be given in the next section. The theory of the apparatus may appear to be somewhat complicated, but actually the determination of the constants takes only slightly longer than it does for the other forms of respirometer. Once this is done, the apparatus is so convenient and accurate in practice that the writer prefers it to any other form for general work.

Constant-volume Differential Manometer

With the object of simplifying the theory of the differential manometer, Dickens and Greville (1933, 1) have very recently developed an ingenious constant-volume differential manometer, which is illustrated both diagrammatically and in detail in Fig. 10. In this instrument the two sides of the manometer are connected by a length of rubber tube, and they can be slid up and down independently in the spring clips *C*. A scale is etched on the left-hand tube, and in addition each tube carries a single mark at the mid-point. In use each mark is kept adjusted to the level of the manometric liquid in its tube by sliding the tubes up or down. The gas volume on each side is thus kept constant. The difference in level of the two sides is then read off on the scale. In this apparatus the bore of the tubes to which the flasks are attached must be made very narrow (less than 0·1 sq. mm.) in order to avoid errors, for the extent to which these tubes are immersed in the water of the thermostat

varies during the experiments. The instrument is intended for use with the Warburg type of shaking apparatus.

As the gas volumes remain constant, the theory is simplified, and the constants are given by formula (1).



(*From* Dickens and Greville, 1933, 1)

Fig. 10

Having described the various available types of manometer, we shall now deal with the various methods by which they may be employed for the measurement of cell respiration.

PART II

METHODS OF MEASURING RESPIRATION

The manometric methods of measuring the oxygen absorption of cells may be divided into two groups: namely, those in which the oxygen uptake is measured directly, and those in which it is obtained by indirect calculation. In the direct methods the vessels are provided with suitable receptacles in which alkali is placed. This absorbs the CO_2 produced by the respiring cells, so that it has no effect on the manometer. The reading of the manometer then gives a direct measure of the oxygen absorption. Any of the three main types of manometer described above may be used for this method. Its chief disadvantages are that it gives only the oxygen absorption, though the CO_2 output also can be obtained by indirect means as shown below; and that it cannot be used with solutions containing bicarbonate, as the *p*H quickly becomes too high owing to absorption of CO_2 from the solution by the alkali. This point is of importance in the case of animal tissues, where the absence of bicarbonate from their environment makes the conditions unphysiological and may produce abnormal effects. In spite of these objections, the direct method is still that most widely used, owing to its simplicity.

In the indirect methods, which have been devised to meet these objections, the CO_2 is not absorbed, and the oxygen uptake and CO_2 output are obtained indirectly as described later, either by making use of the great difference in solubility between oxygen and CO_2 or by the introduction

of reagents into the vessels at various stages of the experiment.

A characteristic difference between the two types of method is that whereas the direct method permits the actual course of the oxygen absorption by the cells to be followed, the indirect methods give the total amount of respiration during some definite period, and do not show any changes of velocity which may occur during that period.

CHAPTER IV

THE DIRECT METHOD

In this method the CO_2 is absorbed by alkali, so that the observed change in the amount of gas in the flask gives the oxygen absorption directly. It may of course be carried out with any of the types of apparatus described above. Various forms of flask are used: Fig. 11 *a* shows the flask (capacity

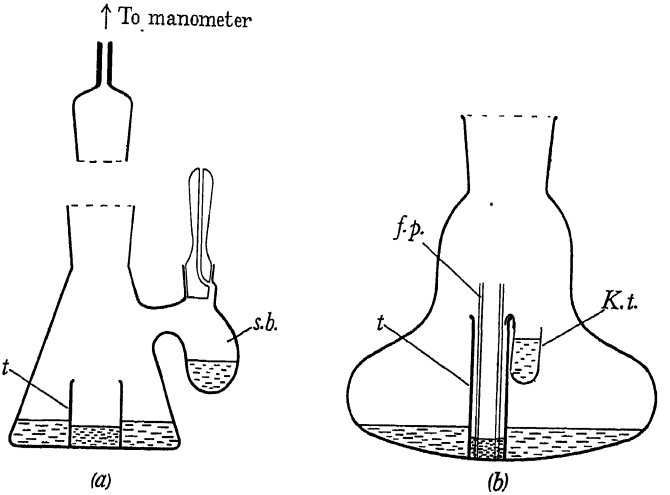


Fig. 11

about 16 c.c.) usually used with the Warburg manometer, and Fig. 11 *b* that used with the Barcroft manometer (capacity about 40 c.c.). The small tubes (*t*) attached to the bottom of the flasks are for the reception of the alkali. The side-bulb (*s.b.*) in Fig. 11 *a* and the small hanging tube (*K.t.*) in Fig. 11 *b* are only used when acid or some other

reagent is to be added during the experiment, as described later, and they are not required for the ordinary determination of oxygen uptake.

Attention must be paid to a number of important points if the results are to represent the true respiration of the tissue or cell suspension, and these will now be considered in turn.

Rate of Diffusion of Oxygen into the Liquid

In the first place, the rate of oxygen uptake must not be too great for the apparatus to deal with. If the amount of tissue is too large the rate of uptake of oxygen will be determined by the rate at which the oxygen can diffuse from the gas space into the liquid, and will therefore not represent the respiration rate of the tissue but will depend on certain purely mechanical factors, such as the rate of shaking and the shape of the flasks. Attention was drawn to this point by Dixon and Tunnicliffe (1923) in connection with the Barcroft apparatus, and the effects have been studied in some detail by Dixon and Elliott (1930). The results of one of their experiments are shown in Fig. 12, which gives the rates of oxygen uptake observed with the Barcroft apparatus using suspensions of yeast of varying concentrations. The abscissae represent the amounts of yeast present in the respirometer, these amounts being in all cases suspended in 3 c.c. of phosphate buffer solution. The experiments were carried out at room temperature with the flasks filled with air. If the results represent the true respiration rate of the yeast the rate of oxygen uptake should of course be proportional to the amount of yeast taken. It will be seen that in the case of curve *A* this is only true up to the point *X*. Below this point the true respiration rate is being measured; above it the oxygen uptake is limited by the rate of diffusion of

oxygen into the liquid, and is almost independent of the amount of yeast. For curve *A* the manometers were shaken at a rate of 102 oscillations per minute. Curve *B* shows the results obtained when the rate of shaking was increased to 138 oscillations per minute, the other conditions being the

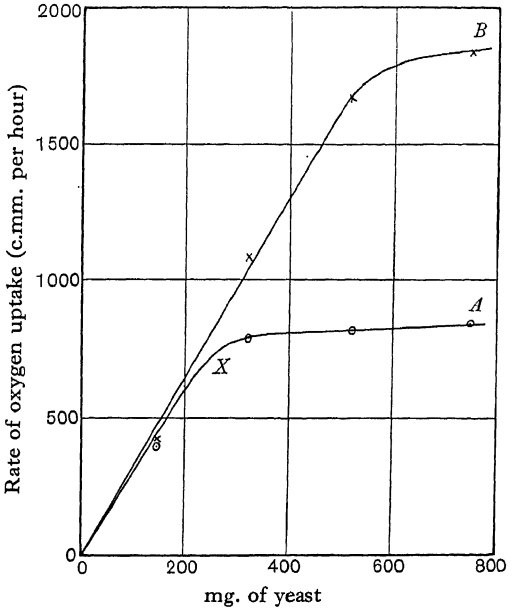


Fig. 12 (*From* Dixon and Elliott, 1930)

same as before. Much higher rates of uptake can now be measured without diffusion effects. It will be seen that below the point *X* the rate of oxygen uptake is independent of the rate of shaking, while above it the rate of shaking becomes the determining factor. Whenever the rate of uptake is found to depend on the rate of shaking this is an

indication that the whole process is being limited by diffusion and that the results are therefore valueless.

It follows from this: (*a*) that a fairly small quantity of tissue should be taken—100 mg. (moist weight) is usually ample in the case of animal tissues and correspondingly less for yeast, etc.—and (*b*) that in measuring a rapid uptake it should always be verified that the rate of uptake is independent of the rate of shaking, and the latter increased if necessary. (It should not be over 200 oscillations per minute with the Barcroft apparatus, as the liquid may tend to splash into the alkali.) The flasks of the Barcroft respirometer have been designed specially to allow free diffusion of gas into the liquid, and Dixon and Elliott find that under the standard conditions of shaking ($2\frac{1}{2}$ cm. stroke; 120 oscillations per minute), and with the flasks filled with air, rates up to about 800 c.mm. per hour could be safely measured, which is ample for most purposes.

Insufficient attention is frequently paid to these points. Experiments have recently been published in which not less than 1·5 *g.* of tissue have been taken, and it is sometimes stated in the literature that it was found necessary to control the shaking carefully in order to obtain consistent results. Such experiments are of little value.

Rate of Absorption of CO_2

The second point to which attention must be paid is that the absorption of the CO_2 by the alkali must be rapid, otherwise considerable errors will be introduced. The production of CO_2 by the respiring tissue may be assumed to take place at a constant rate. The rate of absorption of the CO_2 by the alkali on the other hand will be proportional to the amount of CO_2 in the gas space. A steady state will be

reached when the two rates become equal; and the amount of CO_2 in the gas space will therefore increase until this is the case. This accumulation of CO_2 during the experiment will naturally affect the readings of the manometer and give misleading results. This effect was also studied in detail by Dixon and Elliott (1930), and reference may be made to their paper for a fuller discussion, and for a quantitative investigation on the Barcroft apparatus. They find that the rate of absorption by alkali placed in the small tubes is completely inadequate. The absorption is sufficiently rapid only with "KOH-papers", i.e. small rolls of filter-paper placed in the tubes and soaked with 20 per cent. KOH (*f.p.* in Fig. 11 *b*). About 0·4 c.c. of alkali is usually added to each tube. The volume must of course be measured, and must be taken into account when calculating the constant of the apparatus. The rolls of paper should be long enough to project about 5 mm. above the top of the tube, and they should be of No. 40 Whatman (starch-free) paper. (If ordinary paper is used some oxygen is absorbed when it is soaked with the alkali.) Dixon and Elliott showed that if "KOH-papers" were used the accumulation of CO_2 was negligible provided the rate of production of CO_2 by the cells was not greater than 1000 c.mm. per hour. When measuring respiration by the direct method in the Barcroft apparatus "KOH-papers" should consequently always be used.

Limiting Thickness of Tissue

The next point relates to the preparation of the tissue. Yeast, bacteria, etc. present no special difficulties, for we may simply use a cell-suspension of suitable concentration. With an animal tissue such as liver the case is, however, not so simple. If we divide the tissue finely we run the risk

of damaging too large a proportion of the cells. If on the other hand we work with comparatively large pieces of tissue the oxygen cannot diffuse in through the tissue sufficiently fast, with the result that the cells in the interior of the tissue are completely asphyxiated, and respiration is confined to a thin layer at the surface of the tissue. The value obtained for the rate of respiration per gramme of tissue will then be completely erroneous. Probably the best course is to follow the procedure of Warburg, and work with thin slices of tissue suspended in a suitable medium. Warburg (1923) has worked out the maximum permissible thickness for the tissue slice, and obtains the following equation:

$$d' = \sqrt{8c_0 \frac{D}{A}} \qquad \text{.....(7),}$$

where c_0 is the concentration of oxygen immediately outside the tissue slice (in atmospheres), A is the rate of respiration of the tissue (in c.c. per minute per c.c. of tissue), D is the diffusion constant of oxygen in the tissue substance (in c.c. of O_2 (at N.T.P.) per sq. cm. per minute when the pressure gradient is 1 atmosphere per cm.), and d' is the thickness of the tissue slice (in cm.) at which the oxygen concentration at the centre layer of the slice is just zero. According to the measurements of Krogh, $D = 1{\cdot}4 \times 10^{-5}$ at 38°. A for liver may be taken as 5×10^{-2}, which is slightly above the usual value. If the flasks of the apparatus are filled with air $c_0 = 0{\cdot}2$. The value of d' is then $2{\cdot}1 \times 10^{-2}$. This means that the liver slices must be thinner than 0·2 mm. Now while it is possible (though not easy) to cut slices thinner than 0·2 mm., they are not very suitable for respiration measurements, owing to their great fragility. Uniform slices 0·3 mm. thick, on the other hand, are easily cut, and are quite satis-

factory for such work. But in order to be able to use such slices we must increase the value of d', and we can only do this by increasing c_0. The flasks of the respirometer must therefore be filled with oxygen instead of air, in which case $c_0 = 1$ and $d' = 4{\cdot}7 \times 10^{-2}$. Under these conditions the concentration of oxygen at the centre layer of a 0·3 mm. liver slice is 0·6 atmosphere. As in general the rate of cell respiration remains independent of oxygen pressure until the latter is reduced to a small fraction of an atmosphere, this may be regarded as quite satisfactory.

The easiest way of cutting the slices is as follows. Immediately after removal from the animal a piece of the liver is placed on a filter-paper resting on a flat surface. The upper part is sliced off horizontally with a sharp razor, leaving a uniform piece of tissue about 4 mm. thick adhering to the filter-paper. A number of slices 0·3 mm. thick are cut freehand from the upper surface with the razor, which is kept moistened with Ringer solution. The slices are transferred to a Petri dish containing a little Ringer solution and spread out flat, so that the most suitable ones can be selected. The thickness of a slice can be determined by placing the dish upon squared paper so as to measure the area of the slice, which is then weighed, but after a little practice it is easy to cut uniform slices of the desired thickness. This technique can be applied to many other animal tissues, but several tissues are not sufficiently firm to allow of thin slices being cut.

Filling the Apparatus with Oxygen

The most convenient method of filling the flasks of the Barcroft respirometer with oxygen is the vacuum method of Dixon and Tunnicliffe (1923). The flasks are attached to

the manometer, the taps of which are open, and both sides of the apparatus are connected, by means of thick-walled rubber tubing and a glass T-tube, with a T-tap. The other two branches of this tap are connected with a water-pump and a reservoir of oxygen respectively. Thus by turning the T-tap the apparatus can be alternately exhausted and filled with oxygen. This is usually done twice in succession, the evacuation being taken to a pressure of a few cm. of mercury. A mercury gauge is connected with the tube between the T-tap and the Barcroft apparatus to indicate the pressure within the flasks. The oxygen is kept at a pressure slightly above atmospheric, and an additional tap is provided by means of which the tube leading to the apparatus may be connected with the atmosphere, so as to bring the pressure in the flasks to exactly atmospheric pressure before finally detaching the connecting tubes. The whole process takes only a few seconds. The liquid in the manometer tube remains undisturbed, for the pressure is at every instant the same in both sides of the apparatus, since they are connected together through the rubber tubes and the T-piece.

This method cannot be used with the Warburg apparatus, as one end of the manometer is open to the air. In this case the vessel is filled by passing oxygen through it. The supply tube is attached to the top tube of the manometer, and the oxygen passes through the tap and into the flask, escaping through the outlet provided above the side bulb. The outlet stopper, which is of the type introduced by Warburg and Kubowitz (1929), is so constructed as to act also as a tap, and the outlet may be closed by giving it a half-turn. The current of gas is adjusted so that the manometer shows a pressure of 1–2 cm. of water inside the apparatus, and is

continued for 3–4 minutes, after which the current is stopped, the outlet closed and the supply tube detached.

Medium

The medium in which the tissue is suspended is usually either Ringer solution or phosphate buffer. The writer has found that the same results are obtained whichever is used, at least with yeast and animal tissues, provided the *p*H is the same. Solutions containing bicarbonate cannot of course be used in the direct method, for if the *p*H of such solutions is to be physiological a definite concentration of CO_2 must be present, whereas the direct method only gives correct results if the CO_2 is completely absorbed by the alkali. If it is desired to work with such solutions one of the indirect methods must be used.

Other Practical Details

The most suitable grease to use for the ground joints seems to be anhydrous lanoline, which is sufficiently soft at room temperature and yet does not become too fluid at 40°. When attaching the flasks to the manometers it is best to apply three or four spots of grease with a glass rod, and then to press the flask gently into place without rotating it. The grease then flows uniformly over the ground surfaces, displacing the air and giving a thoroughly reliable joint. If the grease is smeared over the whole surface with the finger, and the flask then worked on by rotating it several times, a certain amount of air will be entangled with the grease and may possibly give rise to leaks later.

A source of error which is frequently met with in manometric work, unless precautions are taken to avoid it, is due to gradual movement of the flasks on the manometer. The

flasks are held in place either by springs or by rubber bands tightly stretched, so that there is always a force tending to pull them more closely on to the manometer. When the apparatus is placed in the warm thermostat the grease becomes softer and gradually flows out of the joint under the action of this force. The flask therefore slowly moves further on to the ground neck, so reducing the internal volume and giving an apparent output of gas, which may in some cases amount to 30 or 40 c.mm. The following precaution completely eliminates this, and should never be omitted. After placing the apparatus in the thermostat the flasks are worked on to their ground joints by rotating them through a few degrees in either direction alternately until the joints bind and become fairly rigid. There is no danger in practice of the joints sticking permanently.

Experimental Procedure

The whole experimental procedure for the determination of the respiration of a tissue by the direct method may be summarised as follows. The correct amount of the medium is first measured into the flasks, and the rolls of filter-paper introduced into the absorption tubes by means of forceps. The animal is then killed, the required tissue removed as quickly as possible and slices cut as previously described. A suitable slice is placed in the liquid in the experimental flask by means of a platinum wire. A measured amount of alkali is then run on to each roll of filter-paper by means of a capillary pipette, care being taken that no trace runs on to the outside of the tubes. The flasks are attached to the manometer and secured by springs or rubber bands. The apparatus is next filled with oxygen by the method described above, and placed in position in the thermostat with the taps

still open. The flasks are then worked on to their ground joints, and the apparatus is shaken in the thermostat until the temperature of the flasks and their contents has risen to that of the bath. Five minutes equilibration is usually sufficient in the case of the Barcroft apparatus, which is insensitive to temperature changes, but 10 to 15 minutes are usually required for the Warburg manometer. (A simple means of determining whether equilibration is complete in the Barcroft apparatus is to close the left-hand tap only. Any movement of the manometer is then due to a change in the temperature of the left-hand flask, and indicates that equilibration is still incomplete.) At the end of this period the taps are closed, and readings are then taken at intervals (e.g. every 10 minutes) for about an hour, the apparatus being shaken the whole time. The rate of respiration usually remains constant during this time, but if the experiment is prolonged much beyond this period the rate may tend to fall off somewhat. At the end of the experiment the taps are opened, the respirometer removed from the thermostat, and the *p*H of the liquid in the flasks tested by adding a few drops of indicator (a precaution which should never be neglected). The tissue slice is removed by means of a platinum wire, washed for a moment in distilled water, placed in a small weighing tube, dried at 110° and weighed. As much grease as possible is removed from the flasks with a cloth; they are then washed out with tap water and immersed for at least 15 minutes in sulphuric-bichromate cleaning mixture (made with concentrated sulphuric acid), after which they are thoroughly washed with tap and distilled water and drained.

The respiration rate is obtained by dividing the amount of oxygen absorbed in a given time by the weight of tissue

taken. It is usually denoted by the symbol Q_{O_2}, which is defined as the number of c.mm. of oxygen (at N.T.P.) per hour per mg. dry weight of tissue. Q_{O_2} is a negative quantity, since the oxygen is absorbed and not evolved.

Determinations of respiration rate on different slices cut from the same organ, e.g. a liver, usually give the same values within 5 per cent.; but determinations on different livers, even of the same species, may show wide variations, depending apparently upon the metabolic state of the animal at the time of death. Determinations on homogeneous cell-suspensions, e.g. of yeast or bacteria, which can be measured with a pipette, are usually found to agree to within 2 per cent.

"*Differential Measurements*"

Before leaving this part of the subject, it may be mentioned that in studying, for instance, the oxidation of a substance by a tissue or cell-suspension the procedure has sometimes been adopted of placing the tissue plus the substance in one flask of the differential manometer and an equal amount of the tissue alone in the other flask. The reading of the manometer is then assumed to give the oxygen absorbed in the oxidation of the substance, since there is an equal amount of tissue on the two sides of the manometer and its own respiration should therefore cancel out as far as the manometric reading is concerned. It should be pointed out, however, that if the two flasks of the apparatus are of different volumes this will not be the case and erroneous results will be obtained. For it is clear from equation (6) that the absorption of x c.mm. of oxygen in the right-hand flask will not produce the same manometric reading as the absorption of x c.mm. in the left-hand flask unless the volumes of the two flasks are the same, so that the respiration of the tissue

itself will only be balanced out when this condition is fulfilled. This point has not always received sufficient attention in the past.

Measurement of CO_2 *Output*

Hitherto we have considered only the determination of the oxygen uptake. We shall now turn to the measurement of the CO_2 production by the direct method. In order to do this two manometers are required, with the same amount of tissue in each. In one manometer the CO_2 is absorbed by alkali in the usual way, so that the reading of this apparatus gives the oxygen uptake directly. In the second manometer the alkali is omitted, so that the CO_2 is not absorbed, and the reading is the resultant of the oxygen uptake and the CO_2 output. Let us suppose that in a given time the amounts of oxygen and CO_2 involved are x_{O_2} and x_{CO_2} respectively. We may perhaps picture the events more clearly by assuming that the CO_2 production and the oxygen absorption take place successively instead of simultaneously. The production of the stated amount of CO_2 will give rise to a manometric reading $h_{CO_2}=\frac{x_{CO_2}}{k_{CO_2}}$. The subsequent absorption of the stated amount of oxygen will then change the reading by an amount $h_{O_2}=\frac{x_{O_2}}{k_{O_2}}$ (h_{O_2} and x_{O_2} are of course both negative quantities). The final reading of the manometer will of course be simply the algebraic sum of these two component readings, so that if h is the reading of this manometer we have

$$h=\frac{x_{O_2}}{k_{O_2}}+\frac{x_{CO_2}}{k_{CO_2}},$$

or

$$x_{CO_2}=\left(h-\frac{x_{O_2}}{k_{O_2}}\right)k_{CO_2} \qquad \text{.....(8).}$$

Now we know x_{O_2} from the reading of the first manometer, and k_{O_2} and k_{CO_2} (the constants of the second manometer for oxygen and CO_2 respectively) can be obtained as described previously. h is observed, so that we can calculate x_{CO_2}.

This method can be carried out with either type of manometer.

If tissue slices are used they are of course dried and weighed at the end of the experiment. In practice, the weights of the two slices will probably not be exactly equal, and it is necessary to take account of this in the calculation. The simplest procedure is to divide the reading of each apparatus by the weight of tissue which was present in it, and to use the resulting values in the calculation. Thus x_{O_2} obtained from the first manometer will be the oxygen uptake per mg. of tissue, and if h is the reading of the second manometer per mg. of tissue, this value of x_{O_2} can be substituted in the equation, and the value of x_{CO_2} then obtained will be the CO_2 output per mg.

Use of Phosphate Solutions or Serum. "Retention" of CO_2

If substances are present which act as buffers in the physiological range of *p*H a new factor is introduced into the determination of the CO_2 output; for they will react with some of the CO_2 produced, converting it into bicarbonate, and this part of the CO_2 will not be shown by the reading of the manometer. This is spoken of as "retention" of CO_2 by the solution. Phosphates and proteins are substances which act in this way; for instance in experiments done in phosphate buffer solution at *p*H 7·4 the following reaction occurs:

$$Na_2HPO_4 + CO_2 + H_2O = NaH_2PO_4 + NaHCO_3,$$

and when serum is used as the medium a similar effect is produced by the serum proteins. In order to measure the total CO_2 of respiration this effect must be eliminated by adding acid to the solution and tissue in the second respirometer at the end of the experiment, so liberating the retained CO_2 once more from the bicarbonate, before taking the final reading. As, however, the tissue and solution will probably already contain a small amount of bound CO_2 at the beginning of the experiment, this necessitates a third respirometer, containing the same amount of tissue and medium as the other two, in which the acid is added to the liquid at the beginning of the experiment immediately after closing the taps. The change of reading of this manometer on adding the acid gives the bound CO_2 initially present, and this must be subtracted from the amount given by the second manometer in order to obtain the amount of CO_2 formed during the experiment. Since the principle of this method was described by Warburg (1914) it has been adopted by many workers.

In the case of the Warburg apparatus the acid is placed in the side-bulb shown in Fig. 11 *a*, and it can then be mixed with the liquid in the flask at any desired time during the experiment by removing the apparatus temporarily from the thermostat and carefully tilting it. (When working at 38° it is necessary to place the finger firmly on the open end of the manometer tube before removing the apparatus from the thermostat, otherwise the Brodie fluid in the manometer will be immediately sucked into the flask by the fall in temperature.) In the Barcroft apparatus the acid is conveniently placed in the small tubes introduced by Keilin (1929). These are provided with small platinum hooks, so that they can be hung from the edge of the absorption tubes

as shown at *K.t.* in Fig. 11 *b*, and they can be subsequently dislodged by a gentle blow on the top of the apparatus. (A gentle blow with the closed hand is more effective than a sharp tap, which may cause some splashing inside the flasks.) These "Keilin-tubes" are very convenient for use whenever a reagent is to be added to the liquid in the flask during an experiment. The acid may be 3*N* HCl or some sufficiently strong organic acid such as oxalic, and enough should be added to make the solution sufficiently acid to stop the respiration of the tissue.

For the determination of the respiratory quotient of tissue slices this method has one rather serious disadvantage, namely that the measurement of oxygen uptake and of CO_2 output are made on two different slices, and the accuracy of the method depends on the two slices having identical respiration rates. It is not always easy to make certain of this, and consequently the accuracy of the CO_2 determination leaves something to be desired. To meet this objection Dickens and Šimer developed their first method.

CHAPTER V

THE FIRST METHOD OF DICKENS AND ŠIMER

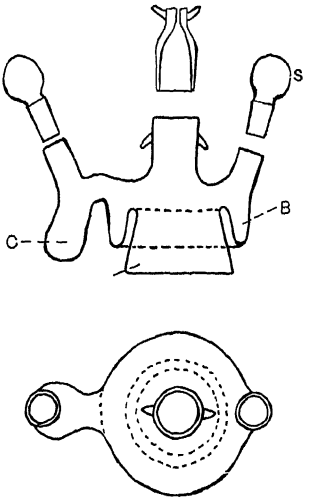
The principle underlying this method was worked out independently by Meyerhof and Schmitt (1929) and by Dickens and Šimer (1930, 1).* The method of Meyerhof and Schmitt was developed primarily for measuring the respiratory quotient of nerve. The apparatus used by Dickens and Šimer is slightly simpler, and appears to be rather more suitable for general work. The method will therefore be described here in the form given to it by Dickens and Šimer.

In this method the measurements of oxygen and CO_2 are both done on the same slice of tissue. The principle of the method is simply that the oxygen uptake is first determined by the direct method, the CO_2 being absorbed by alkali; and at the end of the experimental period the absorbed CO_2 is again liberated by the addition of acid to the alkali as well as to the medium, the resulting change of reading of the manometer giving the total amount of CO_2 finally present. The amount of CO_2 initially present in the tissue and solutions is determined by a second respirometer, containing the same amount of tissue, in which the acid is added at the beginning of the experiment; and this amount is subtracted from the final amount of CO_2 to obtain the amount produced during the experiment. Thus, while two similar slices of tissue are required, one of these merely serves as a control

* Reference should also be made to the recent review by Dickens and Šimer (1933), in which they describe their two methods in detail.

to give the initial CO_2, and both respiratory measurements are done on the other slice. This is a considerable improvement on the previous method.

The special flasks used are illustrated in Fig. 13. They are attached to Warburg manometers of the usual type. The total volume is about 20 c.c. The tissue, suspended in 2 c.c.



(*From* Dickens and Šimer, 1930, 1)

Fig. 13

of the medium (e.g. phosphate-Ringer solution), is placed in the central part *A*. The annular trough *B* contains 0·5 c.c. of a cold-saturated solution of $Ba(OH)_2$ (about *M*/6), and the side-bulb *C* contains 0·3 c.c. of 2·5*N* HCl. By tilting the apparatus the contents of all three compartments can be mixed together, and the acid then liberates the CO_2 which has been absorbed by the barium hydroxide, as

well as any which has been retained by the tissue and medium.

Precautions are taken to keep the CO_2 content of the solutions as low as is conveniently possible. The barium hydroxide solution is kept in a reservoir protected from CO_2, from which it is passed as required through a Berkefeld filter-candle into a burette similarly protected. For the Ringer solution isotonic solutions of NaCl, $CaCl_2$ and KCl are prepared. 100 c.c. of the NaCl, 2 c.c. of $CaCl_2$, 2 c.c. of KCl and 2 c.c. of 10 per cent. glucose are then mixed together, boiled for 15 minutes, allowed to cool in a stream of CO_2-free oxygen, and made up to the original volume with CO_2-free water. To 100 c.c. of this solution is then added 10 c.c. of isotonic *p*H 7·4 phosphate buffer solution, which is kept in a protected burette.* The solution must be thoroughly cooled before adding the phosphate, since the final solution is supersaturated with respect to calcium phosphate; but if the vessels are perfectly clean this usually remains in solution.

Experimental Procedure

In performing an experiment the tissue slices are first suspended in phosphate-Ringer solution for a few minutes while a stream of oxygen (freed from CO_2 by passing through a long tube filled with soda-lime) is passed through. This procedure gives a greater uniformity in the amount of preformed CO_2 in the slices. The acid is then added to the side-bulbs of two flasks. The phosphate-Ringer solution is then quickly measured into the first flask, the tissue slice introduced, the vessel immediately attached to its mano-

* For tissues with a high glycolysis more phosphate should be added, otherwise the lactic acid produced will alter the *p*H too much.

meter and a stream of CO_2-free oxygen passed through it, escaping round the loosely inserted small stoppers. The same procedure is then followed with the second vessel, which should contain the same weight of tissue as the first. The barium hydroxide solution is now added while the stream of gas is still passing, the stopper *S* being momentarily removed. The current of gas is now stopped and the stoppers pushed home at the same time, and the manometers are placed in position in the thermostat. It is important that they should both be transferred to the thermostat at the same time, so that the respiration in both follows the same course. An additional manometer is also required to serve as a thermobarometer. After equilibrating for 10 minutes (but not longer) the first reading of the manometers is taken, and the acid in the second apparatus is immediately thoroughly mixed with the other solutions in the flask. The change of reading of this manometer, after shaking for 15 minutes, multiplied by its constant for CO_2, gives the amount of CO_2 in the solutions and tissue at the beginning of the experimental period. The tissue in the first apparatus is allowed to respire for a suitable period, during which readings are taken at intervals in order to follow the oxygen uptake. The shaking causes the barium hydroxide solution to flow freely round the annular trough, and it is then very efficient in absorbing the CO_2. There is in practice no tendency for it to splash over into the medium containing the tissue. At the end of a definite period, measured from the first reading, this manometer is read, and the acid is immediately mixed with the other solutions in the flask. The final reading is taken after shaking for 15 minutes longer. The difference between the first reading and that immediately before adding the acid, multiplied by

the oxygen constant of this apparatus, gives the oxygen uptake during the experimental period; while the change of reading produced by the addition of the acid, multiplied by the CO_2 constant of the apparatus, gives the total amount of CO_2 finally present, from which the CO_2 produced during the period is obtained by subtracting the initial amount as given by the second manometer. All the readings must, of course, be corrected for any change in the thermobarometer. At the end of the experiment the tissue slices are washed, dried and weighed as usual.

Correction for Unequal Weights of Tissue

In the method as just described the accuracy of the determination of the respiratory CO_2 depends upon having equal weights of tissue in the two respirometers. If the weights are unequal, not only will different amounts of preformed CO_2 be introduced with the tissue, but also different amounts will be formed by the tissue during the preliminary period of equilibration, so that the amount at the beginning of the experimental period given by the second manometer will not represent the true amount initially present in the first manometer. Dickens and Šimer themselves actually use a slightly more complicated method than that given above, involving the use of an additional respirometer similar in every way to the others but containing no tissue. The change of reading on mixing the solutions in this apparatus gives the amount of preformed CO_2 in the *solutions*. Provided the precautions mentioned above have been taken, this is quite small (e.g. 10 c.mm.) and remains very constant. On subtracting this amount from the initial amount as given by the second manometer, the amount contributed by the *tissue* up to the beginning of the experi-

mental period is obtained. If we divide this amount by the weight of tissue in the second manometer, multiply by the weight of tissue in the first manometer and add the amount contributed by the solutions, we shall obviously obtain the true amount present in the first manometer at the beginning of the period, for the amount contributed by the tissue will be proportional to its weight. By this means we can obtain accurate results even when the two slices are of different weights.

It is doubtful, however, whether this extra complication is really worth while in practice. For the total initial CO_2 is in general less than 10 per cent. of that produced during the experiment, and of this 10 per cent. it is found that only about half is contributed by the tissue (the other half being already in the solutions). Now with a little practice it is easy to cut two pieces of tissue of the same weight within 10 per cent. by guesswork, or still better by weighing rapidly on a torsion balance after absorbing the excess fluid with filter-paper. Thus the difference in weight will only produce an error of 0·5 per cent. in the respiratory CO_2 determination, and as the estimated accuracy of the method is about 2 per cent. this error may be regarded as unimportant. Further, the figures quoted by Dickens and Šimer show a 30 per cent. difference between duplicate determinations of pre-formed tissue CO_2 by the more complicated method; and in fact, even though no attempt was made to get constant weights of tissue in their experiments, the simpler method applied to their figures gives the same results within 1 per cent.

The constants of the manometers are, of course, calculated from equation (1) above, but it should be noted that the

solubility of CO_2 in strong electrolyte solutions is less than that in water. The addition of the strong acid has an appreciable effect on the solubility, and Dickens and Šimer find that the solubility of CO_2 in the mixture at 38° is only 0·517 (instead of 0·55). Dickens and Šimer's value must therefore be used for α in calculating the constant.

This method of Dickens and Šimer is very convenient and satisfactory for determining the respiratory quotient of tissues, and is capable of giving very accurate results. (For a large amount of interesting work on animal tissues by this method see Dickens and Šimer (1930, 2)). It is, however, essentially an extension of the direct method, and unfortunately the same limitation applies to it, namely, that media containing bicarbonate cannot be used and the tension of CO_2 approximates to zero. The conditions are therefore in this respect unphysiological for animal tissues, and as recent work has shown that the presence or absence of bicarbonate may profoundly affect the respiratory processes in such tissues, it is essential that their respiration should be studied in media containing the physiological concentration of bicarbonate and in gas mixtures containing the physiological tension of CO_2. This is obviously impossible with the methods hitherto described, and thus arises the importance of the indirect methods now to be described.

CHAPTER VI

THE INDIRECT METHOD OF WARBURG

In this method (see Warburg, 1924)* the tissue is suspended in Ringer solution containing 0·025 *M* bicarbonate (the amount present in normal serum) in equilibrium with a gas mixture consisting of 95 per cent. of O_2 and 5 per cent. of CO_2. The respiratory CO_2 is not absorbed, but advantage is taken of the fact that the solubilities of oxygen and CO_2 are very different. Two ordinary Warburg manometers are required, in addition to the thermobarometer. These manometers are provided with plain rectangular flasks of about 12 c.c. capacity, as illustrated in Fig. 14, provided with outlets for passing the gas mixture. The stoppers for the outlets may conveniently be of the Warburg and Kubowitz type, as illustrated in Fig. 11 *a*. The flasks contain only tissue, bicarbonate-Ringer solution and the gas mixture. Equal amounts of tissue, but unequal amounts of bicarbonate-Ringer solution, are added to the two flasks, 3 c.c. of the solution being added to the first and 8 c.c. to

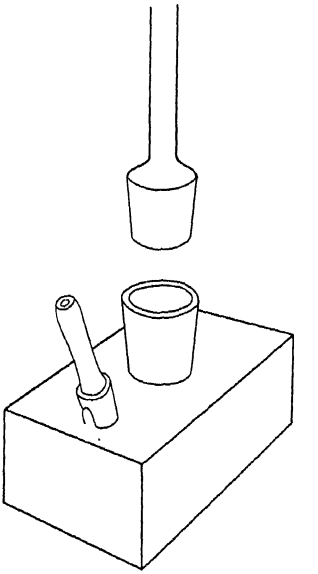


Fig. 14

* A detailed account of the methods developed by Warburg will be found in the review by Krebs (1929).

the second. This is the only difference between the two manometers. Now in the first manometer, in which the gas volume is about three times the liquid volume, the CO_2 constant will be only about 10 per cent. larger than the oxygen constant. But in the second manometer, in which the liquid volume is about twice the gas volume, the CO_2 constant will be perhaps 100 per cent. larger than the oxygen constant, owing to the comparatively large solubility of CO_2. The method depends upon this effect.

Theory of the Method

The oxygen taken up and the CO_2 produced are obtained as follows. The two manometers containing the respiring tissue are shaken side by side, and readings taken at suitable intervals. As the CO_2 is not being absorbed the readings will be in both cases the resultant of the oxygen absorption and the CO_2 production. We have already seen (equation (8)) that under these conditions

$$x_{CO_2} = \left(h - \frac{x_{O_2}}{k_{O_2}}\right) k_{CO_2}.$$

Applying this equation (with small letters) to the first manometer, and writing capital letters for the corresponding quantities in the second manometer, we have for the latter

$$x_{CO_2} = \left(H - \frac{x_{O_2}}{K_{O_2}}\right) K_{CO_2}.$$

If h and H are the respective readings for the same interval of time, and if the respiration rate of the tissue is the same in both manometers, we may write

$$\left(h - \frac{x_{O_2}}{k_{O_2}}\right) k_{CO_2} = \left(H - \frac{x_{O_2}}{K_{O_2}}\right) K_{CO_2}$$

or

$$hk_{CO_2} - HK_{CO_2} = \frac{x_{O_2} k_{CO_2}}{k_{O_2}} - \frac{x_{O_2} K_{CO_2}}{K_{O_2}},$$

whence $$x_{O_2}=\frac{hk_{CO_2}-HK_{CO_2}}{\frac{k_{CO_2}}{k_{O_2}}.K_{CO_2}-K_{O_2}} \qquad \text{.....(9 } a\text{).}$$

And by solving the equations for x_{CO_2} we obtain similarly

$$x_{CO_2}=\frac{hk_{O_2}-HK_{O_2}}{\frac{k_{O_2}}{k_{CO_2}}K_{O_2}-K_{CO_2}} \qquad \text{.....(9 } b\text{).}$$

These two equations enable us to calculate the oxygen uptake and the CO_2 output from the readings of the two manometers. Due attention must of course be paid to the algebraic sign of the various quantities. The four constants are calculated as usual by means of equation (1). The solubility of CO_2 in the Ringer solution used has usually been taken as 0·56 (the same as that in water), but according to the measurements of Hastings and Sendroy (1925) the true value should be 0·537 at 38°, the difference being due to the effect of the dissolved electrolytes.

It is not necessary in practice that the two tissue slices should be of equal weight, provided they are dried and weighed in the usual way at the end of the experiment. By dividing the reading of each manometer by the weight of tissue present in it the reading per mg. of tissue is obtained, and these values may then be substituted in the equations (since the reading will be proportional to the amount of tissue, other things being equal), and they will then give the respiration per mg. of tissue.

Tissue/Medium Ratio

The whole accuracy of the method does, however, depend upon the respiration rates of the two slices (per mg. of tissue) being identical, and any difference will affect both

the oxygen and the CO_2 determinations. Owing possibly to this cause, the accuracy of the method is found to be definitely below that of the other methods. In this connection it may be pointed out that one of the tissue slices is suspended in a much larger volume of liquid than the other. Now it is well known that the effect of washing certain tissues (e.g. muscle) is to remove oxidisable substances and so to lower the respiration; and it might be expected that the tissue slice suspended in the larger volume of liquid, and therefore containing within itself a smaller concentration of any diffusible metabolites, would have a somewhat smaller respiration than the other. This, however, does not appear to be the case with most animal tissues and cell-suspensions. The writer has found that the respiration of a liver slice or of a given amount of yeast is independent of the volume of liquid in which it is suspended within wide limits. In some cases, however (e.g. with slices of heart muscle), it was found that serious errors might be caused by effects of this kind; and, indeed, on attempting to study the oxidation of certain substances by isolated oxidases (for which purpose, it is true, the method was not designed) it was found that the effect of the varying substrate/enzyme concentration relations might be so great as to give an apparent oxygen output instead of an uptake!

The error can be avoided, in cases where it is liable to occur, in two ways. Equal volumes of liquid may be used in two flasks of considerably different volume; or, preferably, the procedure may be as described above, with the exception that the amounts of tissue taken are in proportion to the liquid volumes. Thus since the tissue/medium ratio is the same in both instruments the error is eliminated, and the difference in the amount of tissue can be corrected for by

dividing the reading of each manometer by the corresponding weight of tissue, as described above. In the case of cells such as yeast or bacteria, a homogeneous suspension in bicarbonate-Ringer solution may be prepared and the two different volumes of this measured directly into the flasks, the readings being afterwards corrected as before.

The most suitable amount of tissue to use depends naturally on the respiration rate of the tissue. For animal tissues such as liver from 20 to 50 mg. moist weight is usually taken.

The writer has checked the results given by this method both by parallel determinations, by this and the other methods, of the respiration of yeast (which is not affected by the presence or absence of bicarbonate), and by comparing the observed amount of oxygen absorbed in the oxidation of a known amount of cysteine with the theoretical amount. Satisfactory agreement was obtained in both cases.

In working out the theory of the method no assumption is made as to the kind of respirometer used, and either Barcroft or Warburg manometers may be employed. If Barcroft manometers are used the flasks must be provided with suitable outlets to enable the gas mixture to be passed through, as the vacuum method of filling with gas as previously described cannot be used for mixtures containing CO_2, owing to the fact that a considerable volume of the mixture is required to provide the amount of CO_2 necessary to saturate the liquid at the desired pressure.

Effect of Glycolysis

It is important to remember that the figure for the CO_2 output obtained by this method does not represent the

respiratory CO_2. Many tissues have the power of glycolysis, i.e. the conversion of glucose into lactic acid. Any acid produced by the tissue will liberate a corresponding amount of CO_2 from the bicarbonate of the medium, and the observed CO_2 output represents the sum of the CO_2 produced by the respiration and the CO_2 set free by the glycolysis. The method is therefore not adapted for the measurement of the respiratory quotient.

The usual practice has been to determine the oxygen uptake, and then, *assuming* that the same volume of CO_2 is produced by the respiration (i.e. that the respiratory quotient is 1), to subtract this volume from the total CO_2 output and to take the remainder as the figure for the aerobic glycolysis. This procedure is obviously not very satisfactory, and is at best only an approximation; it has in fact been recently shown by Dickens and Šimer (1931, 2) that the respiratory quotient of animal tissues is frequently considerably below 1, so that many of the published figures for aerobic glycolysis probably require some correction, which is, however, in most cases not very serious. Simultaneous determinations of the anaerobic glycolysis are usually done by having an additional respirometer containing tissue and bicarbonate-Ringer solution, but filled with a mixture of 5 per cent. CO_2 and 95 per cent. nitrogen, freed from traces of oxygen by passage through a heated tube containing copper. In this manometer, of course, no respiration can take place, and the reading, multiplied by the constant for CO_2, gives the anaerobic glycolysis directly. It is convenient to express the glycolysis in the same units as the respiration, i.e. in c.mm. of gas: thus when, following Warburg, we speak of "100 c.mm. of lactic acid" we mean the amount of lactic acid which, if added to a bicarbonate solution,

would liberate 100 c.mm. of CO_2. The symbols $Q_G^{O_2}$ and $Q_G^{N_2}$ may be used to denote the aerobic and anaerobic glycolysis respectively, expressed in c.mm. per mg. dry weight of tissue per hour.* The glycolysis is considered to be a positive quantity if acid is produced, and consequently CO_2 evolved.

A few representative results obtained at 37° by this method are here quoted from Warburg (1926), in order to show the order of magnitude of the quantities. A large amount of data relating to these quantities has been collected in a recent review by Krebs (1933).

Tissue	Q_{O_2}	$Q_G^{O_2}$	$Q_G^{N_2}$	
Liver (rat)	−11·6	+ 0·6	+ 3·3	
Kidney (rat)	−21	0	+ 3·2	
Pancreas (dog)	− 3·2	0	+ 4·2	
,, (rabbit) ...	− 4·6	0	+ 3·4	
Brain (rat) (grey matter)	−10·7	+ 2·5	+19·1	
Retina (rat)	−30·7	+45	+88	
Carcinoma (rat) ...	− 7·2	+25	+31	(mean of 19)
Chick embryo	−10	+ 1·1	+20·6	

The results given by liver are fairly typical of most animal tissues; retina is quite exceptional. With other cells much higher rates of respiration are sometimes obtained: the Q_{O_2} of bakers' yeast may be −250 or more, while with certain bacteria values exceeding −2000 are found. The values for the aerobic glycolysis given in the table are

* The symbols $Q_{CO_2}^{O_2}$ and $Q_{CO_2}^{N_2}$ were formerly used to denote the CO_2 liberated by glycolysis. These were easily confused with Q_{CO_2}, which represents the respiratory CO_2, and they have now been replaced in Germany by the symbols $Q_M^{O_2}$ and $Q_M^{N_2}$ (M=Milchsäure). The symbols suggested above seem to be preferable, as their significance is not limited to one language only.

probably slightly too low, as the respiratory quotient was probably less than 1.

Details of Technique

We may now turn to the practical details of the technique. For the bicarbonate-Ringer solution isotonic (about 0·15*M*) solutions of NaCl, KCl, $CaCl_2$ and $NaHCO_3$ are prepared, and mixed in the proportions of 96 parts NaCl, 2 parts KCl, 2 parts $CaCl_2$ and 20 parts $NaHCO_3$.* 2·4 parts 10 per cent. glucose are also added. The resulting solution contains 0·025 *M* bicarbonate (the concentration present in normal serum) and 0·2 per cent. of glucose (which is also a physiological concentration). Most samples of $NaHCO_3$ are alkaline to phenolphthalein, and on adding such samples to the other salt solutions a precipitate of $CaCO_3$ is produced which is not readily redissolved by passing the CO_2 mixture through the solution. For this reason the $NaHCO_3$ solution should be treated with pure CO_2 until it is just acid to phenolphthalein before mixing it with the other solutions. If this is done no precipitation takes place, and any excess of CO_2 is removed during the subsequent equilibration with the gas mixture.

On exposure of the bicarbonate-Ringer solution to the air CO_2 diffuses out, and $CaCO_3$ precipitates as the solution becomes alkaline. For this reason the solution must be thoroughly saturated with the $CO_2 + O_2$ mixture when it is first prepared, and it must be kept in equilibrium with the gas mixture until required. 150 c.c. bulbs with taps at each

* Krebs and Henseleit (1932) have used a "physiological salt solution" which contains in addition KH_2PO_4 and $MgSO_4$ and corresponds much more closely with mammalian serum than does Warburg's solution.

end, known as gas sampling pipettes, are convenient for this purpose. When any of the solution is withdrawn, a few moments' passage of the gas mixture through the remainder will suffice to keep it in equilibrium.

The gas mixtures can be bought ready prepared in steel cylinders from the usual sources. Their composition should be checked by the usual methods of analysis.

The tissue slices are prepared as previously described. After cutting, they may be suspended for a short time in the solution and treated with a stream of the gas mixture. (Care must be taken to avoid any prolonged exposure to the air, as the solution quickly becomes alkaline through loss of CO_2 and this may affect the tissue.) The correct volumes of solution are meanwhile measured into the flasks from a narrow burette (which should be emptied and washed out as soon afterwards as convenient, in order to avoid deposition of $CaCO_3$ on its walls). The slices are now transferred to the flasks, which are immediately attached to their manometers and placed in the thermostat. The gas mixture is now passed through the flasks for a few minutes with gentle shaking, in order to bring the solution into complete equilibrium with the gas. It is important that this should be done while the flasks are in the thermostat, as the solubility of CO_2 is considerably less at 38° than at room temperature; and if the saturation were done at room temperature, and the manometer then transferred to the thermostat, a considerable amount of CO_2 would be driven off from the liquid into the gas, so giving too high a CO_2 pressure. It is convenient to have the level of the water in the bath so adjusted that the outlet tubes protrude for a short distance above the water; if small bent tubes as shown at *t* in Fig. 17 are attached to these, so that the issuing gas passes through

the water, the control of the operation is facilitated. Care must be taken that the shaking is not sufficiently vigorous to cause any of the solution to be blown out through the outlets, as any loss of fluid would change the constants and might give rise to considerable errors. Loss of fluid by evaporation must also be guarded against by saturating the gas with water-vapour before it enters the manometers, otherwise the passage of a considerable amount of dry gas through the flasks at 38° would cause the liquid volume to be appreciably reduced. This is done by connecting in the supply tube a small bubbling tube containing water and immersed in the thermostat. Except for a sufficient length of thick-walled rubber tubing to allow the apparatus to be shaken while the gas is passing, the gas mixture should be conveyed from the cylinder by means of glass or metal tubes, as the use of rubber tubes would involve too great a loss of CO_2 by diffusion through the rubber. After a few minutes the gas stream is stopped, the outlets and taps closed, and readings taken at intervals. At the end of the experiment the slices are washed, dried and weighed as before.

An ingenious adaptation of the method has been made by Laser (1932) for measuring the respiration of tissue cultures during growth. Instead of carrying out simultaneous measurements on two samples of tissue in different volumes of liquid, only one sample of tissue is used, and the measurements with the two different liquid volumes are made successively in the same respirometer. For this purpose the flask is connected, through a tap, with a bulb of capacity equal to the difference between the two liquid volumes used. The measurement with the larger volume is first carried out in the ordinary way. The tap is then opened, a quantity of medium from the flask is sucked into the bulb

so as to fill it exactly, and the tap closed once more. After a brief equilibration with the gas mixture a measurement is then carried out with the smaller volume of liquid left in the flask. The medium in the bulb may be subsequently run back into the flask, the gas mixture again passed through for a short time, and a further measurement made with the larger liquid volume, and so on. By this means the rate of respiration may be followed over long periods. The original paper should be consulted for full details of the apparatus and method.

*p*H *of the Medium*

The *p*H is maintained sufficiently constant during the experiment by the bicarbonate + CO_2 buffer system. It is determined by the ratio of the CO_2 pressure to the bicarbonate concentration. Now with the usual amount of tissue the glycolysis is not likely to be much greater than 150 c.mm., which is less than 10 per cent. of the bicarbonate initially present in 3 c.c. of the bicarbonate-Ringer solution. The CO_2 constant of the manometer will not be very different from 1, so that the production of 150 c.mm. of CO_2 will produce an increase of about 150 mm. (of Brodie) in the CO_2 pressure. The initial CO_2 pressure is slightly below 500 mm., so that there will be an increase of about 30 per cent., and this, together with the 10 per cent. decrease in the bicarbonate concentration, will cause a decrease of about 0·15 in the *p*H. This represents the largest effect likely to be produced by glycolysis under ordinary conditions. The CO_2 produced by the respiration, with the same assumptions, will also not be much greater than 150 c.mm., and since there is in this case no corresponding decrease in bicarbonate the resulting *p*H change will be slightly over 0·1. High respiration and high glycolysis occur together only in

very rare cases, in which less tissue is required, so that the maximum pH change which is likely to occur is about 0·15, which is usually unimportant.

The initial pH of the solution can be calculated as follows. The pH of a solution containing bicarbonate and dissolved CO_2 is given by the following modified form of the well-known Hasselbalch equation:

$$pH = pK' + \log [NaHCO_3] - \log p_{CO_2} - \log \frac{\alpha}{760 \times 22{\cdot}4},$$

where pK' is a constant, α is the solubility of CO_2 in the solution and p_{CO_2} is the pressure of CO_2 in mm. Hg (see Hastings and Sendroy (1925)). The value of pK' was formerly given as 6·36 at 38°, but Hastings and Sendroy have shown that its value depends upon the ionic strength of the solution, and that its true value in the present case is 6·14. The partial pressure of CO_2 in a gas mixture containing 5 per cent. of CO_2, saturated with water-vapour at 38°, is about 35 mm. Hg; α for this solution is 0·537; and the bicarbonate concentration is 0·025 M. Using these values in the equation we obtain a value of 7·50 for the initial pH.

The question of retention of CO_2 by the medium does not arise when bicarbonate-Ringer solution is used, for it contains no effective buffers other than bicarbonate itself, and therefore cannot bind CO_2 chemically. The small amount of buffering substances contributed by the tissue itself is usually negligible.

Measurements in Serum

(*a*) *Effect of buffers.* In the Warburg method the tissues are in a definitely more physiological environment than in the other methods previously described. It is frequently desired, however, to make the conditions still more physio-

logical by using blood serum itself instead of the bicarbonate-Ringer solution. In this case complications are introduced by the large retention effects which occur with serum, owing to the buffering action of the serum proteins and, to a much smaller extent, the phosphates.

The buffering of a part of the CO_2 increases its effective solubility, and so leads to an increase in the value of the CO_2 constants. To obtain the value of the constant which applies when serum is used we must add a term representing the retention to the normal constant. If we can obtain the true constants for serum we can carry out the experiments precisely as before, and calculate the results by operating with the new constant instead of the normal one.

The buffering action of the serum also affects the results given by the glycolysis, for a certain fraction of the lactic acid produced will be neutralised by the buffers and will therefore be prevented from liberating CO_2 from bicarbonate. There will thus be a "retention of lactic acid" which will affect the value of the constant for glycolysis k_G (k_G is defined as $\frac{x_G}{h_G}$, where x_G is the amount of glycolysis (in c.mm.) which produces a manometric reading h_G). It will be seen later that k_G is not equal to k_{CO_2} when serum is used. The two effects are due to the reactions

$$H_2CO_3 + NaPr = NaHCO_3 + HPr$$

and

$$HL + NaPr = NaL + HPr,$$

where NaPr represents a sodium salt of protein and HL represents lactic acid.

A full mathematical treatment of the whole question is given by Warburg (1925). It is not necessary to give the details here; the principle of the treatment, expressed in

non-mathematical language, is as follows. The cases of respiratory CO_2 and of lactic acid are considered separately. The pH of the serum is assumed to undergo a given small change, owing to the production in the one case of CO_2 and in the other case of lactic acid. Now the amount of acid buffered in bringing about this decrease of pH, which will be equal to the retention in each case, will depend simply on the buffering power of the solution, and can be measured. Moreover, the change of CO_2 pressure corresponding to the pH change can be calculated if the initial CO_2 pressure and bicarbonate concentration, and in the case of lactic acid also the gas and liquid volumes in the vessel, are known. (The pressure changes will be different in the two cases, for if the solution is made more acid by adding CO_2 the bicarbonate concentration will increase, whereas if lactic acid is added the bicarbonate concentration will be diminished; therefore, since the final pH is the same in both cases, the CO_2 pressures must be different.) If then the retention corresponding to the given pH change is divided by the change of CO_2 pressure corresponding to the same pH change, the retention per mm. pressure change of CO_2 is obtained. The retention is approximately proportional to the CO_2 pressure change, at least over a moderate range of pressure, so that if r_C and r_L represent the retention in c.mm. per mm. (Brodie) of CO_2 per c.c. of serum, for the case of CO_2 and lactic acid respectively, and if $k^R_{CO_2}$ is the CO_2 constant when Ringer solution is used, we may write

$$x_{CO_2} = h_{CO_2}(k^R_{CO_2} + v_F r_C) \qquad \text{.....(10 } a\text{)}$$

and

$$x_G = h_G(k^R_{CO_2} + v_F r_L) \qquad \text{.....(10 } b\text{),}$$

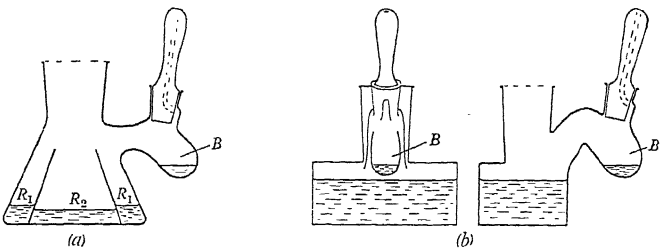
where v_F now denotes the volume of serum in the flask

in c.c. The quantities within brackets are the required constants for use with serum, and they may be written $k^S_{CO_2}$ and k^S_G respectively. Warburg gives methods for the determination of the necessary quantities. This must be done for each sample of serum used, for the constants depend upon the buffering power of the serum, and no two specimens will be identical in this respect. These particular methods will not however be described here, as they have now been superseded by the recent method of Warburg, Kubowitz and Christian (1931), which is much more direct.

(*b*) *Direct measurement of retention.* In this method r_C is first determined for the particular serum used. For this purpose two Warburg manometers with special flasks, as illustrated in Fig. 15 *a*, are required. In one of these 2 c.c. of bicarbonate-Ringer solution is placed in the outer space R_1, and 0·1 c.c. of a lactic acid solution of a suitable strength (say about $M/20$) is placed in the side bulb B; the central space R_2 is left empty. The same solutions are placed in the second flask, and in addition 2 c.c. of the serum is placed in the space R_2. The flasks are then attached to their manometers, placed in the thermostat at 38° and filled with the CO_2+O_2 gas mixture. After proper equilibration the lactic acid is mixed with the bicarbonate solution, thus liberating a definite volume of CO_2, which will be the same in both manometers. In the flask containing serum some of this CO_2 will pass into the serum and be retained there. If h_1, k_1, h_2, k_2 are the readings and CO_2 constants of the first and second manometers respectively (k_2 being calculated on the assumption that the serum does not retain, and is equivalent to an equal volume of bicarbonate-Ringer solution), the amount of CO_2 liberated

will be $h_1 k_1$, and the amount remaining after the amount retained has been taken up by the serum in the second manometer will be $h_2 k_2$. The increase of CO_2 pressure in the second flask is h_2, so that if v_F represents the volume of serum in the second flask in c.c. we have

$$r_C = \frac{h_1 k_1 - h_2 k_2}{h_2 v_F}$$



(*After* Warburg, Kubowitz and Christian, 1931)

Fig. 15

As r_C does not depend on the volumes of gas and liquid present, $k^S_{CO_2}$ can now be obtained for the manometers in which the respiration experiment is to be carried out by means of equation (10 *a*).

On the other hand r_L, as mentioned above, does depend on the gas and liquid volumes, so that if it is determined in any other flask than those actually used in the respiration experiment a somewhat complicated calculation is necessary. For this reason it is very much simpler to determine k^S_G directly in the actual flasks used, rather than to calculate it from equation (10 *b*). This may be readily done if flasks as illustrated in Fig. 15 *b* are used for the respiratory determination. These are the same as those shown in Fig. 14,

with the addition of a side-bulb B, in which is placed 0·1 c.c. of the same lactic acid solution which was used in the previous determination (the lactic acid content of which (in "c.mm.") is therefore known). The respiration experiment is carried out exactly as before, using two manometers with different liquid volumes. At the end of the experiment, however, after a sufficient number of readings has been taken, two readings are taken at an interval of 10 minutes, and immediately after the second the lactic acid is mixed with the serum in both flasks, and another reading taken after a further 10 minutes. The difference between the first and second readings gives the rate at which the reading of the manometers is changing owing to the respiration of the tissue, and if this is subtracted from the difference between the second and third, the result will be the change of reading produced by the addition of the lactic acid. (This procedure is necessary because the tissue continues to respire after the addition of the acid.) This is done for each of the two manometers. Then if x_L is the amount of lactic acid added, and the resulting change of reading of the manometer is h_L, the value of k_G^S for that manometer will be $\frac{x_L}{h_L}$. The constants are thus determined directly. (Full protocols of several experiments are given by Warburg, Kubowitz and Christian.)

(*c*) *Calculation of results.* Having obtained the required constants, we may now consider how they are to be used for calculating the results of the experiments. For the two manometers we have the two equations

$$h=\frac{x_{O_2}}{k_{O_2}}+\frac{x_{CO_2}}{k^S_{CO_2}}+\frac{x_G}{k^S_G},$$

$$H=\frac{x_{O_2}}{K_{O_2}}+\frac{x_{CO_2}}{K^S_{CO_2}}+\frac{x_G}{K^S_G}.$$

(The symbol x_{CO_2} is here used to denote the *respiratory* CO_2, and not, as in equation (9 *b*), the *sum* of the CO_2 of respiration and the CO_2 of glycolysis, for which the symbol x_S will be used from now on. Thus $x_S=x_{CO_2}+x_G$.) It will be seen that we have two equations involving three unknown quantities, and we cannot calculate these quantities separately. We can, however, consider x_S as a single quantity and obtain x_{O_2} and x_S as follows. If the true respiratory quotient of the tissue is represented by R.Q. we may rewrite the equations

$$h=\frac{x_{O_2}}{k_{O_2}}-\frac{x_{O_2}\times \text{R.Q.}}{k^S_{CO_2}}+\frac{x_G}{k^S_G},$$

$$H=\frac{x_{O_2}}{K_{O_2}}-\frac{x_{O_2}\times \text{R.Q.}}{K^S_{CO_2}}+\frac{x_G}{K^S_G};$$

and solving these for x_{O_2} and x_G we obtain

$$x_{O_2}=\frac{hk^S_G-HK^S_G}{\frac{k^S_G}{k}-\frac{K^S_G}{K}} \qquad \text{.....(11 } a\text{)},$$

$$x_G=\frac{hk-HK}{\frac{k}{k^S_G}-\frac{K}{K^S_G}} \qquad \text{...(11 } b\text{)},$$

where $\frac{1}{k}$ has been written for $\left(\frac{1}{k_{O_2}}-\frac{\text{R.Q.}}{k^S_{CO_2}}\right)$ and $\frac{1}{K}$ for $\left(\frac{1}{K_{O_2}}-\frac{\text{R.Q.}}{K^S_{CO_2}}\right)$. We can then obtain x_S from the equation $x_S=x_G-x_{O_2}\times \text{R.Q.}$ Thus in calculating the results we *assume* a value for R.Q., and use the hypothetical value of x_G

thus obtained as an intermediate step in working out x_S. It can easily be shown that the exact value assumed for R.Q. has very little effect on the values of x_{O_2} and x_S finally obtained: practically the same results are obtained no matter whether the R.Q. is taken as 0·7 or as 1·0. It must be clearly understood that the true value of x_G is not calculable, but by assuming a value we are enabled to obtain x_{O_2} and x_S with fair accuracy.

Thus it will be seen that the above procedure enables us to obtain the same results with serum as we can obtain with bicarbonate-Ringer solution, in spite of the retention effects shown by serum. The respiration of animal tissues is usually found to be appreciably greater in serum than it is in bicarbonate-Ringer solution.

The Warburg method has been of great value for the study of animal tissues under conditions made as physiological as possible, and a very large amount of work has been done by means of it, particularly in the investigation of tumours. It has, however, several rather serious disadvantages. In the first place it is impossible to obtain the respiratory CO_2 production, and consequently the respiratory quotient cannot be determined. The glycolysis can be only approximately determined, by assuming a value for the R.Q. In the second place the whole method depends upon the two tissue slices having identical respiration rates, which is a somewhat unsatisfactory feature. And in the third place the use of serum as the medium introduces complications, and the results are obtained by a very indirect method.

In order to eliminate these disadvantages, while retaining the advantages of the Warburg method, Dickens and Šimer devised their second method, which will now be described.

CHAPTER VII

THE SECOND METHOD OF DICKENS AND ŠIMER

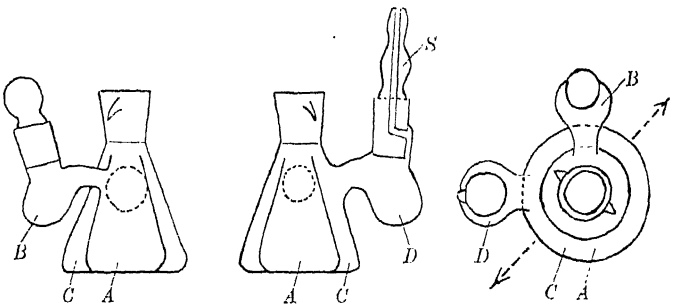
In this method (see Dickens and Šimer (1931, 1; 1933)) the same slice of tissue is used for the measurements of the quantities required, a second slice being used as a control merely to give the initial values. It can be used equally well with bicarbonate-Ringer solution or serum as medium in equilibrium with the $CO_2 + O_2$ mixture, or with phosphate or ordinary Ringer solution as medium with pure oxygen as the gas. Moreover, the oxygen uptake, the *respiratory* CO_2 production and the glycolysis are all independently and accurately determined, and hence the true respiratory quotient of the tissue can be obtained under conditions which are as physiological as possible. The method has therefore many important advantages over those previously described.

Experimental Procedure

For one complete experiment three Warburg manometers, provided with special flasks, are required,* in addition to the thermobarometer. The flasks are illustrated in Fig. 16, and it will be seen that the body of the flask is divided into two compartments, and that two side-bulbs are provided, one opening into each compartment of the flask. The total capacity is about 20 c.c. In the first and second manometers exactly 1·5 c.c. of the medium, together with the tissue, are placed in the central space *A*. (The two tissue slices (of, say,

* These can be obtained from Messrs C. Dixon and Co., 27, Devonshire Street, London, W.C. 1.

100 mg. moist weight) should be made as nearly as possible equal in weight, using a torsion balance for rapid weighing.) This space is left empty in the third flask. In the side-bulb *B* in the first and second flasks is placed 0·1 c.c. of 3*N* HCl. The outer space *C* contains in all three flasks 2 c.c. of *M*/5 $KMnO_4$, containing *M*/500 H_2SO_4, and the bulb *D* in all three flasks contains 0·2 c.c. of 30 per cent. NaI (acidified to *N*/500 with H_2SO_4 immediately before use). Thus at the appropriate time during the experiment the strong acid can be added to the medium and tissue, con-



(*From* Dickens and Šimer, 1931, 1)

Fig. 16

verting the bicarbonate into free CO_2; and on subsequently mixing the iodide with the permanganate a reaction takes place resulting in the formation of a strongly alkaline solution, which then absorbs the whole of the CO_2 from the flask (a principle which is due to Krebs (1930, 1)). The flasks are all filled with the 5 per cent. CO_2 + 95 per cent. O_2 mixture (if bicarbonate-Ringer solution or serum is being used; if not, with O_2) by passing it through as described for the Warburg method. The gas should be passed for at least 10 minutes, as it is essential that the flasks should be completely filled with the mixture.

As the pressure changes in this method are somewhat large it is important that all the stoppers and joints be worked in so that they bind fairly tightly: in fact this is essential for the success of the method. The magnitude of the changes also necessitates the use of a heavier liquid than Brodie solution in the manometer tubes, and Clerici solution* must be used for this purpose. This has a density of about 4, and consists of

50 g. thallium malonate,
50 g. thallium formate,
10 c.c. water.

0·1 per cent. of sodium tauroglycocholate should be dissolved in this; and it is advisable to keep it for a day or two at a temperature slightly below room temperature, and filter, before determining its density. If this is not done it is liable to deposit crystals in the manometer tube. It is also necessary to age the rubber reservoirs of the manometers by previous soaking in Clerici fluid, in order to prevent subsequent blackening of the solution. The liquid does not absorb water spontaneously, but it is advisable to guard against accidental introduction of droplets of water into the manometer tubes, as this would produce a considerable change of density. The open ends of the manometers are therefore protected by means of loosely fitting glass caps, and the moist gas stream is passed to each instrument through a bulb containing loose cotton-wool.

After equilibration, all the manometers are read, and the acid in the first manometer is immediately tipped into the compartment containing the tissue. (Care must be taken while this is being done that none of the iodide comes into

* This can be obtained from Messrs Hopkin and Williams, Ltd., 16, Cross Street, Hatton Garden, London, E.C. 1.

contact with the permanganate.) This manometer is read again after 10 minutes, and once more after a further 5 minutes in order to make certain that the reading has attained its final value. The change of reading on adding the acid measures the initial amount of bicarbonate present. If we call this B_1, and the change of reading H_B, we have $B_1 = H_B K_{CO_2}$. The iodide is then tipped into the permanganate in this apparatus, and the reading taken when it has become constant (after 20–30 minutes). The change of reading on adding the iodide (with its sign reversed) gives the total amount of CO_2 in the flask, including that which was liberated from the bicarbonate. This change of reading (with reversed sign) may be called H_C. The tissue has of course been killed by the acid, so that the readings remain constant, and no correction for any residual respiration is required. The tissue in the second apparatus is allowed to respire for a suitable period from the first reading, say 1 hour, readings being taken at intervals. Let the change of manometric reading during the hour be h. Exactly 1 hour after tipping in the acid in the first flask, the acid is similarly tipped in this flask. Then if the resulting change of reading is h_B, the final amount of bicarbonate is given by $B_2 = h_B k_{CO_2}$. The iodide is then added to the permanganate in this flask, giving a change of reading which (with sign reversed) may be called h_C, and which gives the total amount of CO_2 present at the end of the experiment. (It will be noticed that capital letters are used for the first manometer and small letters for the corresponding quantities in the second manometer.) The CO_2 constants are calculated by means of equation (1) on the assumption that no CO_2 is retained by serum, for the measurements are made after the addition of acid, which liberates any retained CO_2.

Theory of the Method

Now the total amount of CO_2 in the flasks is made up of three parts: (1) the CO_2 which is introduced as a constituent of the gas mixture when the vessel is filled, (2) bound CO_2 (bicarbonate) introduced with the medium and tissue, and (3) CO_2 formed in the respiration. If equal amounts of tissue and medium are added to the two flasks the second and third parts will be the same in both at the time when the first reading is taken and acid added to the first flask. The first fraction, however, will not in general be the same in both, for it forms a definite *percentage* of the gas mixture introduced, and if the flasks are of different volumes, as will usually be the case, it is clear that different *amounts* of CO_2 will be introduced. In order to calculate the results this variable quantity, which depends merely on the dimensions of the apparatus, must be eliminated. This is the purpose of the third manometer, which contains no tissue or medium, but is filled with the same gas mixture as the others. At a convenient time during the experiment the iodide is tipped into the permanganate in this instrument, and the change of reading, which may be called (again with reversed sign) H_{gas}, is observed. This is a measure of the percentage of CO_2 introduced with the gas. It is not necessary, however, to know the constant of this apparatus, or to work out the *volume* of CO_2 introduced. For it is clear that with a given gas mixture at a given barometric pressure the partial pressure will be the same whatever the volume of the vessel, and the change of pressure H_{gas} produced by its absorption is equal to this partial pressure and is therefore the same for all manometers whatever the volume of the flask. The same result is arrived at from equation (1) if we set the

amount of CO_2 equal to a definite fraction of the whole gas. The percentage of CO_2 in the mixture is in fact given by

$$\text{Percentage } CO_2 = \frac{H_{\text{gas}}}{P-p} 100,$$

where P is the initial total gas pressure and p is the vapour pressure of water in the flask (Krebs (1930, 1)). Thus although H_{gas} depends on the barometric pressure it will be the same for all three manometers used in any given experiment. Therefore by subtracting H_{gas}, as determined by the third manometer, from the readings H_C and h_C of the other manometers, the variable quantity represented by fraction (1) is eliminated, and on multiplying the remainder by the CO_2 constant of the apparatus concerned, we obtain the sum of fractions (2) and (3). This quantity, the total CO_2 (bound + free) contributed by the tissue and medium, will from now on be referred to as the total CO_2, and its value at the beginning and end of the experimental period represented by C_1 and C_2 respectively. Thus we have

$$C_1 = (H_C - H_{\text{gas}}).K_{CO_2} \quad \text{and} \quad C_2 = (h_C - H_{\text{gas}}).k_{CO_2}.$$

It is clear that the increase of total CO_2 during the experiment represents the amount of new CO_2 formed, i.e. the *respiratory* CO_2, so that

$$x_{CO_2} = C_2 - C_1 \qquad \ldots\ldots(12\,a).$$

The oxygen uptake is obtained indirectly as follows. The change of reading of the second manometer during the respiration period (h) is the algebraical sum of the oxygen uptake and the change in the amount of free CO_2. (The bound CO_2 naturally does not affect the manometer until it is converted into free CO_2 by acid.) Now the initial free CO_2 (neglecting the amount introduced with the gas, which has been eliminated from the calculations) is the total CO_2

minus the bound CO_2, i.e. is equal to C_1-B_1; and the final free CO_2 is equal to C_2-B_2. The change of free CO_2 is therefore $(C_2-C_1)-(B_2-B_1)$, or $x_{CO_2}-x_B$ (x_B being the change in the amount of bicarbonate, taken as being positive if the bicarbonate increases in amount). We may therefore write

$$h=\frac{x_{O_2}}{k_{O_2}}+\frac{x_{CO_2}}{k_{CO_2}}-\frac{x_B}{k_{CO_2}},$$

and it should be noted that the validity of this equation is quite unaffected by any retention of CO_2 which may occur.

Rearranging this equation we have

$$x_{O_2}=\left(h-\frac{x_{CO_2}-x_B}{k_{CO_2}}\right)k_{O_2} \qquad \ldots\ldots(12\,b),$$

and as all the quantities involved have now been obtained we can calculate x_{O_2}.

When bicarbonate-Ringer solution is used as the medium, the decrease in the amount of bicarbonate is equal to the amount of strong acid produced, i.e. to the glycolysis, so that

$$x_G=-x_B \qquad \ldots\ldots(12\,c).$$

The three quantities required are therefore related to the manometric readings and constants as follows:

$$x_{O_2}=\left[h-\frac{(h_C-H_{gas})k_{CO_2}-(H_C-H_{gas})K_{CO_2}-(h_Bk_{CO_2}-H_BK_{CO_2})}{k_{CO_2}}\right]k_{O_2}$$

$$x_{CO_2}=(h_C-H_{gas})k_{CO_2}-(H_C-H_{gas})K_{CO_2}$$

$$x_G=H_BK_{CO_2}-h_Bk_{CO_2}.$$

The first and second equations are valid for any medium, and are not affected by retention of CO_2 or glycolysis; the third is only valid for bicarbonate-Ringer solution.

Measurement of Glycolysis in Serum

When serum is used, the glycolysis is no longer equal to the change in the amount of bicarbonate, for the retained part of the respiratory CO_2 is converted into bicarbonate, and x_B includes this as well as the glycolysis. A part of the lactic acid is also retained, and this is not included in x_B. In order to determine the glycolysis in serum, the procedure given more recently by Dickens and Šimer (1932) may be used. This involves a calculation of the total retention as follows. A "retention curve", relating change in retention to change in pH, is first constructed for the serum used, by adding varying amounts of acid to the serum (in equilibrium with the CO_2+O_2 mixture), measuring the amounts of CO_2 evolved, and subtracting them from the amounts which would be evolved if there were no retention. The change of pH occurring during the respiration experiment is then calculated from the equation

$$\Delta p\mathrm{H}=\log\frac{B_2}{B_1}+\log\frac{H_C-H_B}{h_C-h_B}.$$

The retention corresponding to this pH change is then read off from the curve, and this is the retention occurring during the actual experiment. Calling this amount R, the glycolysis is given by

$$x_G=R-x_B \qquad \ldots\ldots(12\,d).$$

Reference must be made to the original paper for full details of the procedure and protocols of experiments.

The whole method of Dickens and Šimer works very well in practice, and gives very satisfactory results. The writer has carried out many parallel determinations, comparing it with the other methods, and has obtained very close agree-

ment. It requires, however, careful manipulation and close attention to detail. For instance, the presence of a very slight trace of grease on the inside of the flask may make it very difficult to run the acid quantitatively into the medium, and as the acid is not in large excess considerable errors may be caused. (The amount of acid might be increased with advantage.) It is essential that the flasks are kept scrupulously clean. The MnO_2 which is formed in the flasks may be readily removed by a solution of sodium sulphite + acetic acid, and the flasks must be immersed in strong cleaning mixture until every trace of grease is removed. (This should of course always be done, whatever method is being used.)

The method has many important advantages over those previously described. Its disadvantages are that the determinations involve a rather large amount of experimental work. Four manometers are required for a single experiment. The calculation of the results is also somewhat complicated and indirect. These disadvantages are, however, far outweighed by the special advantages which the method offers.

CHAPTER VIII

THE METHOD OF DIXON AND KEILIN

This method (see Dixon and Keilin (1933)) is based upon the method of Dickens and Šimer just described, but with a considerable simplification brought about by the application of the principle of compensation used in the differential type of manometer. In this method only one differential manometer is required for a complete experiment, and the results are obtained directly without any complex calculation. It can be used for any purpose for which the Dickens and Šimer method can be used, with the exception of the determination of the aerobic glycolysis in serum, for which the method has not yet been adapted.

A Barcroft manometer, provided with two special flasks of equal volume,* is used. One of the flasks is shown in Fig. 17. It has a capacity of about 40 c.c., and consists of a flask of the type usually used with Barcroft manometers, but provided with means for introducing acid and alkali during the experiment. (It may be pointed out that these flasks may, if desired, be attached to Warburg manometers and used instead of Dickens and Šimer's flasks for their method.) Before describing the principle of the method itself, the construction and use of the flasks will be considered.

The medium and tissue are placed in the main part of the flask. The side-bulb contains acid, which can be run into the medium by giving the bulb a half-turn, the bulb

* This can be obtained from Messrs W. G. Flaig and Sons, 57, Hatton Garden, London, E.C. 1.

being fitted to the flask by a ground joint for this purpose.* The tap *T* is for the purpose of introducing alkali at the end of the experiment. The bore of the tap does not pass completely through the barrel, but is closed at one end, forming a cup of about 0·5 c.c. capacity. The method of filling is as follows. The tap is first greased, preferably with a rubber grease, inserted into position, and turned so that the opening of the cup is downwards. The flask is then inverted, the cup completely filled with 40 per cent. KOH by means of a fine pipette, and the tap turned so as to enclose the alkali without including any air bubbles (Fig. 17 *a*). The excess of alkali is removed from the outer tube. The flask is then placed upright in a suitable stand, and a roll of No. 40 Whatman filter-paper (*f.p.*) is inserted with forceps into the tube as shown. The paper should project about 5 mm. above the top of the tube, and it should be opened out so that it fits the inside of the tube fairly tightly and pushed to the bottom of the tube. A piece of glass rod (*r*) (see below) is then placed in the tube inside the filter-paper roll. (When the tap is later turned into the position shown in Fig. 17 *b*, the glass rod drops down into the cup, the alkali is thereby displaced upwards, soaks the filter-paper, and rapidly and quantitatively absorbs the CO_2 from the flask.) The side-bulb is now placed in position, 0·3 c.c. of 3*N* HCl measured into it, and the stopper *s* inserted as shown. A small glass tube as shown at *t* is loosely attached to the upper end of the stopper by means of a short piece of rubber tubing. This is of such a length that when the apparatus is placed in position in the water-bath its open end dips below the surface of the water.

* Repeated control experiments have shown that, provided the joints are worked in initially, no change of volume can be detected on turning the bulb.

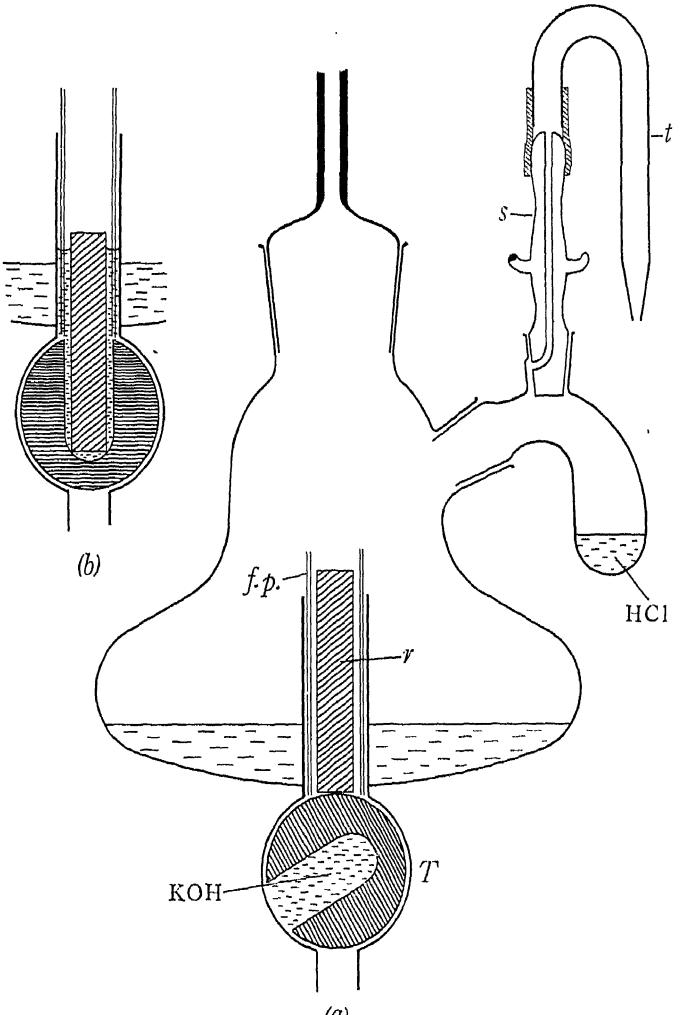
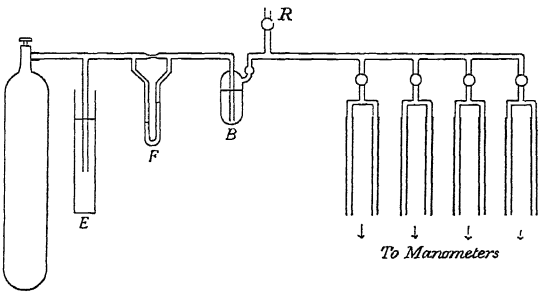


Fig. 17

The joints and taps are now secured by rubber bands stretched fairly tightly.

The second flask is filled similarly at the same time. Exactly equal amounts of the medium (3·0 c.c.), previously brought into equilibrium with the gas mixture, are now measured into the two flasks from a micro-burette; and equal amounts of tissue, weighed on a torsion balance, are added to both flasks. These are immediately attached to the manometer, which contains Clerici solution, and placed in



(*From* Dixon and Keilin, 1933)

Fig. 18

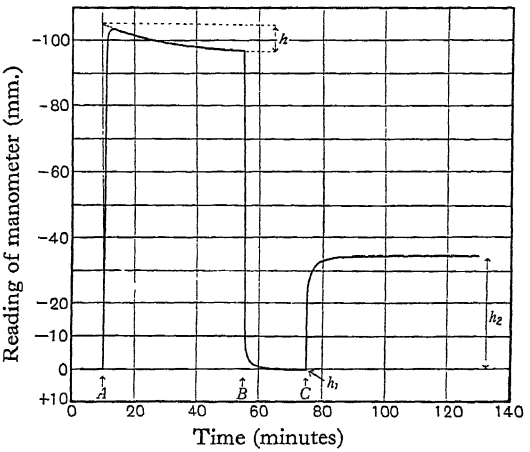
the thermostat. The gas mixture is then passed through the flasks while the apparatus is shaken. For this purpose the arrangement shown in Fig. 18 is used. *E* is a trap containing water to act as an escape for the gas in case the pressure accidentally becomes too great, *F* is a flowmeter for adjusting the rate of flow of the gas, and *B* is a bubbling tube containing water and immersed in the thermostat, the function of which is to saturate the gas with water-vapour before it enters the flasks. Connection is made to the manometers through the branched tubes shown by means of lengths of thick-walled rubber tubing attached to the open

ends of the manometer tubes. The gas is passed through both flasks simultaneously, escaping by way of the stoppers *s*. The taps of the Barcroft apparatus must be adjusted so that the gas passes through the two flasks at approximately the same rate. The gas supply is adjusted by means of the flowmeter so that about 200 c.c. per minute pass through each flask, and this is continued for 10 minutes, as it is essential that both flasks shall be completely filled with the mixture. Meanwhile all the joints are worked in and made to bind fairly firmly, with the exception of the bottom taps, in which case it is neither necessary nor practicable to carry the process as far as with the other joints. At the end of 10 minutes the tap *R* (Fig. 18) is opened, the gas supply stopped, the stoppers *s* closed and worked in, and the small tubes removed from their upper ends. The apparatus is now shaken for a further 5 minutes with the rubber supply tubes still attached, in order to allow complete equilibration and to ensure that the initial pressure is exactly the same in both flasks; after which the taps of the manometer are closed, and the rubber tubes detached.

Up to this point there is no difference between the two flasks, and the manometric reading is of course zero. The description of the subsequent experimental procedure may be made clearer by reference to Fig. 19, which shows the actual manometric readings during the course of an actual experiment on 12 mg. of yeast in bicarbonate-Ringer solution. (Yeast was used as a convenient material for comparison of the methods with one another, and in this experiment parallel determinations were made by the second Dickens and Šimer method, and of the oxygen uptake in phosphate by the direct method.)

After it has been verified that the manometric reading is

zero, the time is noted and the acid in the left-hand flask is run into the medium by turning the side-bulb without removing the apparatus from the bath (point *A* on the curve). This kills the tissue in the left-hand flask, and converts the bicarbonate into free CO_2, causing a positive pressure in the left-hand side of the apparatus. It is not necessary to read the manometer at this stage if the respira-



(*From* Dixon and Keilin, 1933)

Fig. 19

tion only is to be measured; but if the glycolysis also is required two or three readings at 5-minute intervals should be taken. This is in order to extrapolate back to obtain *h*, the change of reading during the experimental period due to the tissue in the right-hand flask. This extrapolation is necessitated by the fact that the CO_2 in the left-hand flask is not evolved quite instantaneously on adding the acid. The figure shows clearly the method of obtaining *h*, which is

only necessary if the glycolysis is to be measured. After allowing the respiration to proceed for a definite period (45 minutes in this case), the acid is run into the medium in the right-hand flask (point *B* on the curve), and the manometer read after a further 15 minutes' shaking (by which time the reading is quite constant). Let h_1 represent this reading. The state of affairs is now the same in both flasks, except that the tissue in the right-hand flask has respired for 45 minutes longer than that in the left-hand flask. The alkali is now introduced into both flasks simultaneously by turning the taps (point *C* on the curve), and after allowing 45 minutes for the absorption of the CO_2 to reach completion, a final reading is taken. Let this be h_2. (It should be made clear that h_1 and h_2 are the actual readings of the manometer, and not the *change* of reading as in the Dickens method.) If tissue slices are used they are of course dried and weighed as usual.

Theory of the Method

It will be seen that the left- and right-hand flasks correspond with the first and second manometers of the method of Dickens and Šimer. In that method the quantities used in the calculation of the results were the differences between them, which with the differential manometer are given directly by the readings. The use of a thermobarometer becomes unnecessary, since the effects of any external changes are eliminated by compensation. Moreover H_{gas} will be the same in both flasks, and will therefore not affect the manometer, so that its determination by a separate manometer is also unnecessary. The method thus becomes greatly simplified.

A corresponding simplification occurs also in the calculation. Consider the final reading h_2. Initially the same amounts of CO_2 (bound and free) were present in both flasks. The whole of this has now been absorbed. Since the flasks are of the same volume the removal of these equal amounts of CO_2 will produce the same pressure change on both sides, and the manometer will remain unaffected. Any fresh CO_2 formed in the respiration has been removed again by absorption; and it is therefore clear that no change of any kind in the CO_2 which may have occurred during the experimental period can have any effect on h_2. Thus h_2 simply represents the oxygen absorption by the tissue in the right-hand flask during the period, and

$$x_{O_2} = h_2 k_{O_2} \qquad \text{.....(13 } a\text{).}$$

At the time of taking the reading h_1 all the CO_2 is present in the free form, and between the taking of the readings h_1 and h_2 this total CO_2 has been absorbed in both flasks. Any change in the reading of the manometer clearly represents the difference between the amounts absorbed in the two flasks, this being the change of total CO_2 during the period, which is obviously the respiratory CO_2. Thus

$$x_{CO_2} = (h_1 - h_2).k_{CO_2} \qquad \text{.....(13 } b\text{).}$$

We can therefore obtain the oxygen uptake and the true CO_2 output, even in serum, in a single respirometer with only two readings.

The change in the amount of bicarbonate is also obtained as follows. It has already been shown, in dealing with the Dickens and Šimer method, that the change of manometric reading during the period of respiration is related to the

oxygen uptake, the CO_2 production and the change of bicarbonate as follows:

$$h=\frac{x_{O_2}}{k_{O_2}}+\frac{x_{CO_2}}{k_{CO_2}}-\frac{x_B}{k_{CO_2}},$$

and substituting the values of x_{O_2} and x_{CO_2} given by the two previous equations we obtain

$$h=h_2+(h_1-h_2)-\frac{x_B}{k_{CO_2}}$$

or

$$x_B=(h_1-h).k_{CO_2}.$$

If the medium is bicarbonate-Ringer solution, the glycolysis is given by $x_G=-x_B$, so that in this case

$$x_G=(h-h_1).k_{CO_2} \qquad \text{.....(13 } c\text{).}$$

The three required quantities are thus simply and directly obtained.

When serum is used the glycolysis is not equal to the change in bicarbonate, and a more complicated procedure would be necessary to determine it. Such a procedure, however, has not yet been worked out. The determination of the O_2 and CO_2 of respiration is equally valid for all the media.

The significance of the three readings may be summarised as follows:

$$h=h_{O_2}+h_{CO_2}+h_G,$$
$$h_1=h_{O_2}+h_{CO_2},$$
$$h_2=h_{O_2}$$

(the first for non-retaining solutions only).

Example

In the experiment represented in Fig. 19 the values of the readings were

$h=+7{\cdot}5$ mm., $h_1=+0{\cdot}1$ mm. and $h_2=-34{\cdot}1$ mm.

The value of k_{O_2} was 13·88, and k_{CO_2} was 14·58. Then

$$x_{O_2} = -34{\cdot}1 \times 13{\cdot}88 = -473 \text{ c.mm.},$$

$$x_{CO_2} = [+0{\cdot}1 - (-34{\cdot}1)] \times 14{\cdot}58 = +498 \text{ c.mm.},$$

$$x_G = (+7{\cdot}5 - 0{\cdot}1) \times 14{\cdot}58 = +108 \text{ c.mm.}$$

The values obtained, compared with those given by simultaneous determinations by the other methods, are shown in the following table.

Method	*Dickens and Šimer*	*Dixon and Keilin*	*Direct Barcroft*
Medium	Bicarbonate-Ringer solution		Phosphate
Gas	O_2+CO_2	O_2+CO_2	O_2
Amount of yeast	6 mg.	12 mg.	12 mg.
x_{O_2}	−232	−473	−471
x_{CO_2}	+252	+498	—
R.Q.	1·08	1·05	—
Q'_{O_2}	− 51·6	− 52·5	− 52·4
x_G	+ 50	+108	—

(Q'_{O_2} represents c.mm. of oxygen per hour per mg. moist weight.)

Further Details of the Technique

A few points relating to the apparatus call for mention. It is essential that the two flasks of each apparatus should have exactly the same volume: if not, the compensation will not be perfect, and serious errors may be introduced into the determination of x_{CO_2} (though they will not affect x_{O_2}). The volumes, including of course the top tubes of the manometer, should not differ by more than 100 c.mm. It is, however, not a matter of great difficulty to adjust the volumes during manufacture even to this degree of accuracy, as the final adjustments can be made by slightly expanding or contracting the side-bulbs. In the instruments which have

been supplied to the writer the differences have been less than 60 c.mm.

The bore of the manometer tubes should be somewhat larger than usual (between 0·6 and 1·5 sq. mm.), in order to allow the Clerici solution to flow quite freely. This does not introduce errors into the value of the constant, as with a heavy liquid the correction terms in the formula become relatively much smaller. It may also be pointed out that the use of this liquid does not in practice cause any loss in sensitivity, as the readings themselves become correspondingly more definite and precise. The liquid keeps for long periods in the manometers without change, as it is not in contact with rubber in this method.

The constants are calculated from formula (6) in the usual way. In obtaining v_G, the volume of the glass rod as well as v_F, the volume of the medium plus that of the HCl, must be subtracted from the total volume of the flask, which must not include the cup of the bottom tap. In preparing these rods a number of equal lengths are cut from the same rod, ground down till they are all of the same weight, and rounded off in the flame. They will then be interchangeable, as they will be all of the same volume, which volume can be found with sufficient accuracy by measuring the diameter and length of one of them. Mercury was at first used for displacing the alkali from the taps, but the glass rods have been found to be more satisfactory.

Sufficient time must be allowed for complete absorption of the CO_2 by the alkali at the end of the experiment before taking the final reading, and neglect to do so may give rise to serious errors. Not less than 45 minutes should be allowed, although the absorption is usually complete before this.

When starting to use the method, or when testing a new

apparatus, several blank experiments should first be done. These are carried out exactly as described above, except that no tissue is added to the flasks. In this case h_1 and h_2 should obviously be zero, and it is actually found in practice that they do not exceed $\pm 0{\cdot}3$ mm., corresponding to an experimental error of ± 5 c.mm. A greater value than this indicates some fault in the apparatus or procedure.

It may be found that the weights of the tissue slices are not quite identical in the two flasks, but unless the difference is large the error due to this cause will be negligible, as also in the second Dickens and Šimer method; for one of the slices is used only as a control to give the initial values, and the amount of CO_2 it contributes is only a small fraction of the whole.

The method gives a considerable saving of time, compared with the unmodified method of Dickens and Šimer. The flasks take less time to fill, fewer readings have to be taken, the calculation is much simpler, and several manometers can be shaken simultaneously in the thermostat.

In conclusion, it may be mentioned that the method is useful also for studying the oxidation of substances added to the tissue or tissue preparation. The tissue is placed in both flasks as before, and the substance is placed in the right-hand flask only in a small hooked tube as previously described. This is dislodged after the equilibration period so as to fall into the medium. The acid is not run into either flask until the end of the experimental period, when it is run into both flasks simultaneously. Otherwise the procedure is the same as before. The result then obtained gives the oxygen uptake and CO_2 output due to the added substance, already corrected for the spontaneous respiration of the tissue itself.

The Method of Dickens and Greville

Dickens and Greville (1933, 2) have very recently* applied the method of Dixon and Keilin to their own constant-volume differential manometer, described on p. 40. The principle and theory of their method need not be described, as they are identical with those of the Dixon and Keilin method, and the results are calculated by means of the equations (13 *a*, *b* and *c*) already given. The manipulations are, however, different in several respects.

The flasks used resemble those used in the second method of Dickens and Šimer, illustrated on p. 88, except that the side-bulb *B* is replaced by a small receptacle fixed to the bottom of the central portion *A*. This receptacle is so shaped that by tilting the flask in one direction the liquids in *C* and *D* may be mixed without mixing the liquid in the receptacle with that in *A*, while by tilting the flask in the opposite direction the latter two may be mixed together. The authors now prefer these flasks for the Dickens and Šimer method, instead of the original form. The tissue and medium are placed in the outer portion *C*, instead of in *A* as previously, the acid is placed in *D*, the iodide in *A* and the permanganate in the inner receptacle.

The manometer tube contains Brodie solution, the procedure having been modified so as to make this possible.

The experiments are carried out as follows. The solutions and tissue are placed in the two flasks, which are then attached to the manometer and filled with the CO_2+O_2 mixture as before. The apparatus is then equilibrated for a short period with the taps open to the air, after which the experimental flask is connected with the manometer by

* This section was added while the book was in proof.

means of its T-tap, the other T-tap being turned so as to close off the control flask completely and to open the manometer tube to the air on this side. The tap T (Fig. 10) is then closed in order to immobilise the manometric liquid, the limb of the manometer tube carrying the control flask detached from the clips C, and tilted so as to mix the acid with the medium and tissue in this flask. After replacing this side of the apparatus the tap T is opened, and the manometer shaken for 1 or 2 hours, readings being taken at intervals in order to determine h. As the end of the manometer tube is open to the air during this period it is necessary to have a separate thermobarometer, and to correct the readings accordingly. During the whole of the experimental period there is a considerable pressure inside the control flask, owing to the liberation of CO_2 by the mixing of the acid with the bicarbonate, but as this flask is completely closed off this pressure cannot yet affect the manometer. It is this which allows the heavy manometric liquid to be dispensed with.

At the end of the experimental period the experimental flask is also closed off by turning its T-tap, and the acid mixed with the medium, using the same procedure as for the control flask. Both flasks are now closed systems, and both limbs of the manometer tube are open to the air, so that the reading is of course zero. After a period of shaking, both T-taps are turned simultaneously so as to connect the flasks to the manometer. The resulting reading is of course h_1. The permanganate is now tipped into the iodide in both flasks, and the final reading obtained after 1 hour's shaking is h_2.

It will be seen that the essential difference between this method and Dixon and Keilin's method (apart from the

difference of apparatus) is the closing off of the flasks by taps before the acid is tipped. This allows the use of Brodie solution in the manometer, as only the comparatively small *differences* of pressure now have to be measured, and the sensitivity of the method is consequently increased (although, for the reasons given on p. 106, the increase in sensitivity is not as great as might be expected from the diminution in density of the manometric liquid). It also allows a much larger amount of bicarbonate to be used than was permissible in the Dickens and Šimer method, which is an advantage if tissues with a high glycolysis are to be used. The amount of bicarbonate now used is of the same order as that used in the Dixon and Keilin method, and may be considered to be ample for all ordinary purposes. A disadvantage of this procedure is that it necessitates the use of a separate thermobarometer if the glycolysis is to be measured.

As some doubt is expressed in Dickens and Greville's paper as to the reliability and freedom from leaks of Dixon and Keilin's ground-in bulbs (although they did not actually test them), it may be stated here that the present writer has always found them to be perfectly reliable, and has never experienced any trouble of this kind. On the other hand it will be clear, in view of what has been said on p. 36 above on the subject of T-taps, that the taps of the Dickens and Greville manometer must be very carefully ground, as they are to withstand the full pressure involved during the whole period of the experiment without danger of leaking. Dr Dickens has, however, informed the author that he has found them to be quite reliable.

CONCLUSION

The main methods have now been described. Each has its own advantages and disadvantages. The direct method will naturally be preferred, on account of its simplicity, when the oxygen uptake alone is required and when the experiments need not be done in bicarbonate solutions. When the CO_2 output is also required under the same conditions the first method of Dickens and Šimer will be used. Both these methods show the actual course of the oxygen uptake throughout the experiment; the latter gives also the amount (but not the course) of the CO_2 production during the experiment. The Warburg method enables the oxygen (but not the CO_2) to be measured in bicarbonate solutions, and by working out the results for a number of successive periods the course of the reaction can be followed. Both the oxygen and the CO_2 of respiration can be measured in bicarbonate solutions by the second method of Dickens and Šimer, or more directly by the method of Dixon and Keilin, and these two methods may be used also for measurements in the absence of bicarbonate instead of the first Dickens and Šimer method. They give, however, only the amounts of oxygen and CO_2 reacting during the experiment; the course cannot be followed, and any changes of respiration rate or of respiratory quotient which may occur during the experiment are not recorded. No method has yet been devised which will permit the course of oxygen uptake and of CO_2 output to be followed in bicarbonate solutions. These two methods are to be preferred to the Warburg method when serum is used as the medium, as they are not affected by retention by the serum.

All the methods assume that no gas other than O_2 and

CO_2 is produced or absorbed. This is generally true except in the case of bacteria. Certain bacteria, however, are liable to give off hydrogen and other gases, and in these cases the methods fail.

Manometric methods are used for many purposes other than the measurement of cell respiration. They have been much used for the study of oxidations brought about by oxidases and other catalysts, as well as for investigating the reactions of haemoglobin and other blood pigments and for the measurement of blood gases. Any reaction in which a gas is absorbed or evolved can be conveniently and accurately studied by these methods. But their usefulness can be still further extended, for studying reactions in which acid or alkali is produced or used up, by causing them to take place in bicarbonate solutions in equilibrium with a gas mixture containing CO_2, and measuring the amount of CO_2 set free or absorbed. This principle was used, for example, by Lipmann and Meyerhof (1930) in order to follow the initial *p*H changes which occur when a muscle contracts; by Krebs and Donegan (1929) to study the hydrolysis of peptides; by Krebs (1930, 1, 2) to follow the enzymatic hydrolysis of proteins; by Reid (1931) to measure the reduction of methylene blue by bacteria; and by other workers. The action of urease can also be studied manometrically by measuring the CO_2 produced from the urea (Kubowitz and Haas (1933)), and Krebs and Henseleit (1932) have made use of this in their classical work on the formation of urea in liver tissue. The manometric technique used in all these cases does not, however, call for any special comment here, as it consists simply in the application to various purposes of the methods already described in this book. The adaptability of the manometric technique is very great, and its use is constantly being extended.

APPENDIX I

DETAILS OF CALIBRATION OF AN ACTUAL BARCROFT DIFFERENTIAL MANOMETER BY TWO METHODS

(1) *By use of formula* (6).

Required: k_{O_2} for use at 38° when $v_F = v_F' = 3$ c.c. and $\cos\theta = 0{\cdot}935$.

(*a*) Determination of P_0.

Weight of 10 c.c. paraffin (in specific gravity bottle previously calibrated with mercury) = 7·880g. Therefore its density = 0·788, and

$$P_0 = \frac{760 \times 13{\cdot}60}{0{\cdot}788} = 13110 \text{ mm.}$$

(*b*) Determination of A.

Length of Hg drop in left side of U-tube = 97·9, 98·3 mm. at 21°.

Length of Hg drop in right side of U-tube = 98·7, 99·1 mm. at 21°.

Weight of Hg drop = 508·2 mg.

Therefore $$A = \frac{508{\cdot}2}{13{\cdot}54 \times 98{\cdot}5} = 0{\cdot}381 \text{ sq. mm.}$$

(*c*) Determination of v_G, v_G'.

Weight of Hg filling left flask and tubes = 487·1 g. at 18°.

Weight of Hg filling right flask and tubes = 488·2 g. at 18°.

Therefore $$v_G' = \frac{487{\cdot}1 \times 1000}{13{\cdot}55} - 3000 = 32950 \text{ c.mm.}$$

$$v_G = \frac{488{\cdot}2 \times 1000}{13{\cdot}55} - 3000 = 33030 \text{ c.mm.}$$

(*d*) Calculation of k_{O_2}.

From formula (6):.

$$k_{O_2} = \left(0{\cdot}935 + \frac{0{\cdot}381 \times 13110}{2 \times 32950}\right) \times \left(\frac{33030 \times \frac{273}{311} + 3000 \times 0{\cdot}024}{13110} + \frac{0{\cdot}381 \times 273}{2 \times 311}\right)$$

$$= 2{\cdot}409.$$

(2) *By Münzer and Neumann's method.*

Temperature of calibration = 18·5°, and barometric pressure = 759 mm. Hg.

Previous calibration of the graduated gas pipette showed that the readings required to be corrected by multiplication by the factor 1·020.

No. of determination	Pipette reading (c.mm.)	Manometer reading (mm.) Left	Right	Difference	h'		k_C' (uncorr.)
(1)	40	103·3	104·1	−0·8			
	245	66·3	141·5	−75·2	−74·4	−205	2·755
(2)	42	103·7	104·5	−0·8			
		74·8	133·7	−58·9	−58·1	−160	2·754
(3)	42	103·9	104·6	−0·7			
	318	53·6	154·4	−100·8	−100·1	−276	2·757
(4)	43	103·7	104·7	−1·0			
	258	64·6	143·5	−78·9	−77·9	−215	2·760
						Mean =	2·756

The value of k_{O_2} for 38° is then given by

$$2{\cdot}756 \times 1{\cdot}020 \times \frac{273}{311} \times \frac{759 - 16}{760}$$

$$= 2{\cdot}412.$$

APPENDIX II

TABLES OF PHYSICAL CONSTANTS

Table I. *Reduction of gas volumes to* N.T.P

The gas is assumed to be measured, saturated with water-vapour, at the temperature and pressure shown in the table. To obtain volumes of dry gas at 0° C. and 760 mm. Hg, multiply the observed volume by the figure shown, which includes the correction for water-vapour.

Temp.	Total Pressure (gas + water-vapour) (mm. Hg)						
° C.	720	730	740	750	760	770	780
10	0·903	0·915	0·928	0·941	0·953	0·966	0·979
15	0·882	0·894	0·907	0·919	0·932	0·944	0·957
20	0·860	0·873	0·886	0·898	0·910	0·922	0·935
25	0·839	0·851	0·863	0·875	0·887	0·899	0·911
30	0·816	0·827	0·839	0·851	0·863	0·875	0·887
35	0·791	0·802	0·814	0·825	0·837	0·849	0·860
40	0·763	0·774	0·786	0·797	0·809	0·820	0·832

Table II. *Density of mercury*

In g. per c.c.

Temp. ° C.	Density	Temp. ° C.	Density
0	13·596	22	13·541
14	13·561	24	13·536
16	13·556	26	13·531
18	13·551	28	13·527
20	13·546	30	13·522

Table III. *Vapour-pressure of water*

In mm. Hg

Temp. ° C.	Vapour-pressure	Temp. ° C.	Vapour-pressure
10	9·2	30	31·7
15	12·8	35	42·0
20	17·5	40	55·1
25	23·7	45	71·6

Table IV. *Solubilities of gases*

The solubility (α) is expressed as c.c. of gas (at N.T.P.) dissolved by 1 c.c. of water when the pressure of the gas is 760 mm. Hg.

Temp.	10°	15°	20°	30°	40°
CO_2	1·194	1·019	0·878	0·66	0·53
N_2	0·020	0·018	0·016	0·014	0·012
O_2	0·038	0·034	0·031	0·026	0·023

At 38°, $\alpha_{O_2}=0{\cdot}024$; $\alpha_{N_2}=0{\cdot}0122$; and $\alpha_{CO_2}=0{\cdot}55$ for water, 0·537 for Ringer solution, and 0·517 for Ringer solution containing 0·3 *N* HCl.

Table V. *Conversion factors*

Some authors express their results in mg. of O_2, or in c.c. of *M*/100 O_2 solution, instead of in c.mm. at N.T.P., which is the standard way. To convert these results into c.mm., multiply by the factors given in the following table ("c.mm. of lactic acid" are as defined on p. 73):

To convert:	O_2	CO_2	Lactic acid
mg. to c.mm.	700	509	249
c.c. of *M*/100 solution to c.mm.	224	224	224

REFERENCES

ADAM (1920). *Biochem. J.* **14**, 679.
BARCROFT (1908). *J. Physiol.* **37**, 12.
—— (1914). *Respiratory Function of the Blood.* Appendix I. Cambridge University Press.
DICKENS and GREVILLE (1933, 1). *Biochem. J.* **27**, 213.
—— —— (1933, 2). *Biochem. J.* **27**, 1479.
—— and ŠIMER (1930, 1). *Biochem. J.* **24**, 905.
—— —— (1930, 2). *Biochem. J.* **24**, 1301.
—— —— (1931, 1). *Biochem. J.* **25**, 973.
—— —— (1931, 2). *Biochem. J.* **25**, 985.
—— —— (1932). *Biochem. J.* **26**, 90.
—— —— (1933). Abderhaldens *Handbuch.* Abt. IV, Teil 13, 435.
DIXON and ELLIOTT (1929). *Biochem. J.* **23**, 1182.
—— (1930). *Biochem. J.* **24**, 820.
—— and KEILIN (1933). *Biochem. J.* **27**, 86.
—— and TUNNICLIFFE (1923). *Proc. Roy. Soc. Lond.* B **94**, 266.
HALDANE (1921). *J. Path. Bact.* **23**, 443.
HASTINGS and SENDROY (1925). *J. Biol. Chem.* **65**, 445.
KEILIN (1929). *Proc. Roy. Soc. Lond.* B **104**, 206.
KREBS (1929). Oppenheimer: *Die Fermente*, **3**, 635.
—— (1930, 1). *Biochem. Z.* **220**, 250.
—— (1930, 2). *Biochem. Z.* **220**, 283.
—— (1930, 3). *Biochem. Z.* **220**, 289.
—— (1933). Oppenheimers *Handbuch d. Biochem.* Ergänzungsbd. I, 863.
—— and DONEGAN (1929). *Biochem. Z.* **210**, 7.
—— and HENSELEIT (1932). *Z. physiol. Chem.* **210**, 33.
KUBOWITZ and HAAS (1933). *Biochem. Z.* **257**, 337.
LASER (1932). *Biochem. Z.* **251**, 2.
LIPMANN and MEYERHOF (1930). *Biochem. Z.* **227**, 84.
MEYERHOF and SCHMITT (1929). *Biochem. Z.* **208**, 445.

MÜNZER and NEUMANN (1917). *Biochem. Z.* **81**, 319.
REID (1931). *Biochem. Z.* **242**, 159.
WARBURG (1914). *Z. physiol. Chem.* **92**, 231.
—— (1923). *Biochem. Z.* **142**, 317.
—— (1924). *Biochem. Z.* **152**, 51.
—— (1925). *Biochem. Z.* **164**, 481.
—— (1926). *Über den Stoffwechsel der Tumoren.* Berlin: Springer. (Eng. trans. by F. Dickens, 1930. London: Constable.)
—— and KUBOWITZ (1929). *Biochem. Z.* **214**, 7.
—— KUBOWITZ and CHRISTIAN (1931). *Biochem. Z.* **242**, 170.
WINTERSTEIN (1912). *Biochem. Z.* **46**, 440.

INDEX

CAMBRIDGE: PRINTED BY WALTER LEWIS, M.A., AT THE UNIVERSITY PRESS